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Inhibition by Chloramphenicol of Aminoazo Dye Carcinogenesis in Rat Liver: RNA Synthesis in Isolated Liver Nuclei*

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Abstract—*RNA synthesis has been measured in isolated rat liver nuclei following pair-feeding of diets containing* 0·06% *3'-methyl-4-dimethylaminoazobenzene (3'MeDAB),* 2% *chloramphenicol (CAP) or both* 0·06% *3'MeDAB and* 2% *CAP for periods of* 4 *and* 10 *days. At* 4 *days, there were significant increases in* $Mn^{2+}/(NH_4)_2SO_4$*-stimulated activity in rats fed CAP or both 3'MeDAB and CAP and a significant increase in the* Mg^{2+}*-stimulated activity in rats fed 3'MeDAB and CAP relative to the control rats. After* 10 *days of pair-feeding,* Mg^{2+}*-stimulated RNA synthesis was increased in all the treated groups, being significantly greater in the group receiving both 3'MeDAB and CAP than in the group receiving 3'MeDAB alone and* $Mn^{2+}/(NH_4)_2SO_4$*-stimulated RNA synthesis was significantly increased in the group receiving CAP. α-Amanitin was shown to inhibit* 88·6% *of the* $Mn^{2+}/(NH_4)_2SO_4$*-stimulated activity and* 20·6% *of the* Mg^{2+}*-stimulated activity. Loss of RNA during the* Mg^{2+}*-stimulated incubation was noted and this was less in the group receiving both 3'MeDAB and CAP than in the other groups, but incubation at* 17°C*, which prevented the RNA loss, revealed that the differences in incorporation between the groups were not a result of differential loss of RNA during incubation. The RNA/DNA ratio of the isolated nuclei was increased in rats fed CAP and was greater in rats fed both 3'MeDAB and CAP than in rats fed 3'MeDAB alone. The effect of altering the concentration of* Mg^{2+} *in the nuclear isolation medium on subsequent incorporation* in vitro *was measured; maximal incorporation in the* Mg^{2+}*-stimulated reaction was noted if nuclei were isolated in* 10*mM* Mg^{2+}*, while the* $Mn^{2+}/(NH_4)_2SO_4$*-stimulated reaction was maximal if* 3*mM* Mg^{2+} *was used.*

INTRODUCTION

THE PRECISE mechanism of chemical carcinogenesis is unknown and therefore studies with inhibitors of this process are of use in determining which of the alterations induced by carcinogen administration are essential for tumour induction. Chloramphenicol (CAP) has been shown to inhibit carcinogenesis in rat liver by 4-dimethylaminoazobenzene [1] and 3'-methyl-4-dimethylaminobenzene (3'MeDAB) [2] and, in the latter study, this inhibition was associated with a normal RNA/DNA ratio in contrast to the depressed RNA/DNA ratio following 3'MeDAB administration. Although the DNA content of the liver increased at a similar rate in rats fed either 3'MeDAB or a diet containing both 3'MeDAB and CAP, an increase in liver RNA content was delayed in rats fed 3'MeDAB, but took place in parallel with the DNA increase in rats fed both 3'MeDAB and CAP [3]. The differences in total liver RNA content between rats fed 3'MeDAB or 3'MeDAB plus CAP may reflect differences in rates of RNA synthesis and degradation between these groups. In the present study, the

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capacity of isolated liver nuclei from rats pretreated with either 3'MeDAB or 3'MeDAB plus CAP to synthesize RNA *in vitro* has been investigated, as it has been reported [4] that nuclear RNA synthesis measured *in vitro* provides an accurate means of assessing RNA synthesis in growing and non-growing cells.

Widnell and Tata [5] found evidence that nuclei incubated in the presence of Mg^{2+} synthesize ribosome-like RNA while those incubated in the presence of Mn^{2+} and $(NH_4)_2SO_4$ synthesize DNA-like RNA. There are also findings which suggest that these reactions are catalyzed by 2 different polymerase enzymes located in the nucleolus and the nucleoplasm respectively [6]. More recently, Roeder and Rutter [7] have isolated 2 major RNA polymerase activities from rat liver nuclei; I, which is located in the nucleolus [8] and is probably necessary for the synthesis of ribosomal RNA [9] and II, which is located in the nucleoplasm [8] and might be necessary for the synthesis of DNA-like RNA. However, it has been found that $(NH_4)_2SO_4$ stimulates RNA synthesis in the isolated nucleolus [10] and inhibits soluble RNA polymerase II [8]. In addition, Yu and Feigelson [11] reported opposing effects of $(NH_4)_2SO_4$ on RNA synthesis in nuclei; on the one hand there was evidence that it may interfere with RNA polymerase–template complex formation while on the other, there was evidence for increased DNA transcription due to salt-mediated dissociation of repressor proteins from the chromatin complex. In the present study, incubations were performed in the presence of either Mg^{2+} or Mn^{2+} and $(NH_4)_2SO_4$ as described by Widnell and Tata [5], but because of doubt as to whether these reactions represent the activities of polymerases I and II of Roeder and Rutter [7], some incubations were performed in the presence and absence of α-amanitin, which has been reported to inhibit specifically nucleoplasmic RNA synthesis catalyzed by RNA polymerase II [12].

MATERIALS AND METHODS

Animals and diets

Male Sprague–Dawley rats (127–172 g) were obtained from random-bred departmental stock. Groups of 4 animals were housed one to a cage and pair-fed a control diet and diets containing 2% CAP, 0·06% 3'MeDAB and 0·06% 3'MeDAB plus 2% CAP as described previously [3] for periods of 4 and 10 days. All animals were killed between 0900 and 1000 hours.

Source of materials

3'MeDAB (m.p. 119°C) was synthesized as described by Giese *et al.* [13] and chloramphenicol (D(-)*threo* isomer) was a gift from Parke, Davis and Co., Caringbah. The following materials were purchased: ATP (disodium salt), GTP (trisodium salt), CTP (disodium salt), UTP (sodium salt) and calf thymus DNA from the Sigma Chemical Co., St. Louis; uridine-4-^{14}C-5'-triphosphate (specific activity 50 mCi/m-mole and 46 mCi/m-mole) from the Radiochemical Centre, Amersham and α-amanitin from C. H. Boehringer Ingelheim through Calbiochem, San Diego.

Isolation of nuclei

The method used was basically similar to method B of Widnell and Tata [14], but the underlay step prior to the low-speed centrifugation was omitted and the quantities of materials used were varied so that nuclei were isolated from individual livers. The rats were exsanguinated under light ether anaesthesia and the livers removed and placed in ice-cold homogenizing medium (0·32M sucrose, 3mM $MgCl_2$). All subsequent procedures were carried out at 0–4°C. Liver samples (4 g) were homogenized (10 strokes, 1150 rev/min) in 12 ml of homogenizing medium in a Potter–Elvehjem homogenizer with a teflon pestle (size B, A. H. Thomas and Co., Philadelphia) and the homogenate was filtered through a single layer of nylon tulle; the homogenizing vessel and tulle were then rinsed with an additional 4 ml of homogenizing medium which was added to the homogenate. The combined filtrate was centrifuged at 750 ***g*** for 10 min in a Sorvall RC2-B centrifuge and the resultant crude nuclear pellet suspended in 2·4M sucrose, 1mM $MgCl_2$ (8 ml) and centrifuged at 50,000 ***g*** for 1 hr in a Spinco Model L ultracentrifuge using the 65 rotor. The nuclear pellets obtained were resuspended in 1 ml of 0·25 M sucrose containing 1 mM $MgCl_2$. In one experiment, where an attempt was made to determine whether the state of aggregation of liver nuclei affected the rate of RNA synthesis, the concentration of $MgCl_2$ in the 2·4 M sucrose was varied between 1 mM and 10 mM.

Assay of nuclear RNA synthesis

The incubation media described by Widnell and Tata [5] were used for the assay of Mg^{2+}-stimulated and $Mn^{2+}/(NH_4)_2SO_4$-stimulated RNA synthesis in isolated nuclei; each tube contained 0·3–0·7 mg of DNA and 0·255 μC (0·005 μ-mole) of ^{14}C–UTP. The incubation medium for the Mg^{2+}-stimulated reaction

contained in a final volume of 0·5 ml: Tris–HCl (pH 8·5), 50μ-mole; $MgCl_2$, 2·5μ-mole; cysteine, 10μ-mole; NaF, 3μ-mole; GTP, CTP and ATP, 0·3μ-mole; unlabelled UTP, 0·015 μ-mole, ^{14}C–UTP and nuclear suspension (0·1 ml). The reaction was initiated by the addition of the nuclei. The incubation medium for the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction contained in a final volume of 0·5 ml: Tris–HCl (pH 7·5), 50μ-mole; $MnCl_2$, 2μ-mole; $(NH_4)_2SO_4$ (adjusted to pH 7·5 with NH_4OH), 203 μ-mole; GTP, CTP and ATP, 0·3 μ-mole; unlabelled UTP, 0·015 μ-mole, ^{14}C–UTP and nuclear suspension (0·1 ml). Nuclei were preincubated at 37°C for 15 min and the reaction initiated by the addition of ^{14}C–UTP; α-amanitin was added where indicated at zero time following preincubation. All incubations were performed in duplicate. In initial experiments, Mg^{2+}-stimulated activity was measured by incubating under air at 37°C for 15 min, however, as significant RNA loss was noted during incubation and the peak of incorporation occurred earlier in our studies, routine measurements were made after incubating for 6 min. In some experiments, duplicate samples were incubated at 37°C or 17°C for various intervals up to 20 min. The $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity was measured by incubating under air at 37°C for 15 min or for 45 min after a 15 min preincubation. The reactions were terminated by the addition of ice-cold 0·3 M $HClO_4$ (1 ml), the precipitate collected by centrifugation at 2°C and washed twice with 0·2 M $HClO_4$ (3 ml). The pellets were hydrolyzed with 0·3 M KOH, then the DNA and protein were precipitated with 0·6 M $HClO_4$ and collected by centrifugation as described by Blobel and Potter [15]; the final volume of the supernatant containing hydrolyzed RNA was 2·9 ml. An aliquot (1 ml) of this solution was added to 15 ml of a scintillation mix comprising 2:1 toluene/Triton X-100 containing 4 g PPO/l prior to liquid scintillation counting and water (1 ml) was added to the residual solution prior to determining the absorbance at 260 nm. Absorbance was converted to mg RNA by assuming that an extinction of 1·00 at 260 nm was given by a solution containing 32 μg RNA/ml from rat liver [16]. The residual pellet was dissolved in 0·1 M KOH (5 ml) and an aliquot (0·1 ml) diluted to 2 ml with water prior to DNA analysis [15]. Radioactivity measurements were made with a Packard Model 3003 liquid scintillation spectrometer. By using this procedure, analysis of incorporated radioactivity and DNA and RNA content were made on zero time and incubated samples and it was possible to determine whether there was loss of DNA or RNA during incubation.

Microscopic observations

The nuclear suspensions were diluted 1 part in 20 with 0·25M sucrose containing 1mM $MgCl_2$ and nuclear counts were performed in a Spencer haemocytometer with a Neubauer ruling. Smears were prepared from the undiluted nuclear suspensions, stained with Leishman's stain and examined under the light microscope.

Determination of DNA and RNA

Nuclear DNA and RNA were estimated as described above by a procedure which was basically similar to that described by Blobel and Potter [15]; homogenate DNA and RNA were determined using the procedure described by Munro and Fleck [17].

Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test except where otherwise indicated.

RESULTS

Nuclear preparations from rats which had been pair-fed diets containing CAP, 3'MeDAB or both 3'MeDAB plus CAP showed significant increases in incorporation of ^{14}C–UTP into nuclear RNA; in particular, nuclei from rats fed the diet containing 3'MeDAB and CAP were more active in this respect than nuclei from rats fed 3'MeDAB (Table 1). In these initial experiments, where samples were incubated as described by Widnell and Tata [5], it was noted that there was significant loss of RNA during the Mg^{2+}-stimulated reaction, although little, if any loss of RNA took place during the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction (Table 1); there was no loss of DNA during incubation in either reaction. Furthermore, RNA loss in the Mg^{2+}-stimulated reaction was significantly less ($P < 0·05$) in the group receiving both 3'MeDAB and CAP than in the group receiving 3'MeDAB (Table 1) and consideration was given to the possibility that this difference might account for the difference in incorporation of ^{14}C–UTP between these groups. In view of this finding, and because an inverse relationship has been noted between nuclear ribonuclease activity and UMP incorporation into RNA in isolated nuclei [18], a time study was conducted to determine whether the loss of RNA during the Mg^{2+}-stimulated reaction influenced the kinetics of the reaction and was in any way responsible for the differences in incorporation

Table 1. RNA synthesis and % loss of RNA on incubation in isolated rat liver nuclei obtained from rats pair-fed a control diet and diets containing CAP, 3'MeDAB or 3'MeDAB plus CAP for 4 days

Group	Mg^{2+}-stimulated activity			$Mn^{2+}/(NH_4)_2SO_4$-stimulated activity		
	pmoles ^{14}C-UTP incorporated/ 100 μg DNA	% Difference from control	% RNA loss on incubation	pmoles ^{14}C-UTP incorporated/ 100 μg DNA	% Difference from control	% RNA loss on incubation
Control	13·0 ± 7·7 (7)	—	16·3 ± 8·2 (8)	22·6 ± 15·5 (6)	—	−1·1 ± 6·9 (6)
CAP	23·7 ± 5·3 (7)	+ 83·0*	17·9 ± 5·5 (8)	33·3 ± 9·3 (6)	+47·6†	2·3 ± 3·2 (6)
3'MeDAB	16·4 ± 3·6 (6)	+ 26·3	19·6 ± 7·7 (7)	24·9 ± 7·4 (5)	+10·5	5·8 ± 4·2 (5)
3'MeDAB plus CAP	41·2 ± 5·8 (7)	+217·4*‡	11·0 ± 5·7 (8)	37·3 ± 6·3 (6)	+65·4†‡	1·9 ± 3.0 (6)

The assay tubes were incubated at 37°C for 15 min in the case of the Mg^{2+}-stimulated reaction, and for 45 min after a 15 min preincubation at 37°C in the case of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction. Results are presented as the mean ± S.D. Numbers in parentheses indicate the number of experiments.

*Significantly different from the control group as determined by Student's *t*-test ($P < 0·02$, two-tailed test).

†If, during processing the data, each value was expressed as a % difference from the respective control value and statistical analysis was performed on this basis, these results were significantly different from the control group ($P < 0·05$, Student's *t*-test, one-tailed).

‡The differences in incorporation between nuclei from rats fed 3'MeDAB or 3'MeDAB plus CAP were significant (Mg^{2+}-stimulated activity, $P < 0·01$, $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity, $P < 0·05$, using the two-tailed Student's *t*-test).

between the experimental groups shown in Table 1. This study revealed that on incubation at 37°C, maximal incorporation was observed after 6 min in all experimental groups and that thereafter there was a decrease (Fig. 1). It seemed likely that both the loss of RNA during incubation and the decrease in incorporation after 6 min incubation were a consequence of nuclear ribonuclease activity, which has been assayed under conditions identical with those used in the Mg^{2+}-stimulated reaction [18–21] but is inhibited during the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction [18, 21]. Incubations were therefore performed using conditions (incubation at 17°C) which have been reported to inhibit the activity of degradative enzymes [22]. Under these conditions the differences in incorporation between the experimental groups were still apparent when RNA loss during the Mg^{2+}-stimulated reaction was not significant and incorporation of ^{14}C-UTP into RNA was linear with time for 10 min (Fig. 1). In addition, it can be seen in Fig. 2 that at 38°C, decreasing incorporation of ^{14}C-UTP into RNA was associated with increasing loss of RNA in the nuclei, while at 17°C, there was a linear increase in incorporation for 10 min and negligible RNA loss. Furthermore, when a time study was made of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction, where RNA loss was insignificant, incorporation of ^{14}C-UTP increased and reached a plateau, but did not decrease. These findings suggested that nuclear ribonuclease activity was responsible for the decrease in incorporation in the Mg^{2+}-stimulated reaction and therefore all further samples were incubated for 6 min only at 37°C. In the case of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction, it was noted that plateau levels of incorporation were reached by 15 min in nuclei from control rats, whereas prolonging the incubation time in treated nuclei revealed further increases in incorporation; the data reported here were obtained from either 15 or 45 min incubations, as indicated.

Although the absolute values of Mg^{2+}-stimulated ^{14}C-UTP incorporation into RNA in isolated rat liver nuclei were lower after 10 days of pair-feeding than after 4 days in all experimental groups (Fig. 3a), incorporation in all treated groups was relatively greater than in the control group (Fig. 3b) and these relative increases were slightly greater at 10 days than

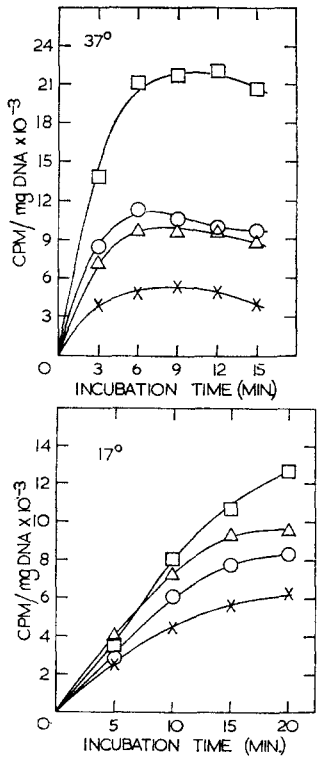


Fig. 1. Effect of incubation temperature on incorporation of ^{14}C–UTP into RNA (Mg^{2+}-stimulated, pH 8·5) in isolated nuclei from rats fed control diet, × — ×, or diets containing CAP, △ — △, 3′MeDAB, ○ — ○ or 3′MeDAB plus CAP, □ — □. Each point represents a single analysis on a separate aliquot of nuclear suspension derived from one rat on each of the dietary regimens.

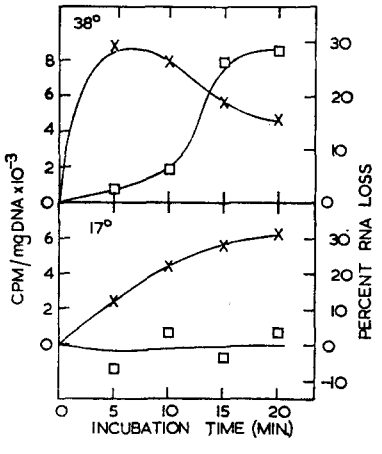


Fig. 2. Effect of incubation temperature on the kinetics of ^{14}C–UTP incorporation into RNA (Mg^{2+}-stimulated, pH 8·5) × — × and % RNA loss in liver nuclei □ — □ from a rat receiving the control diet. Each point represents a single analysis on a separate aliquot of nuclear suspension. Similar results were obtained in each of the 3 experimental groups.

after 4 days of pair-feeding. The decreases noted in the absolute values are possibly a consequence of the decrease in food intake of the rats due to the pair-feeding regimen as there are reports that diet restrictions result in decreases in

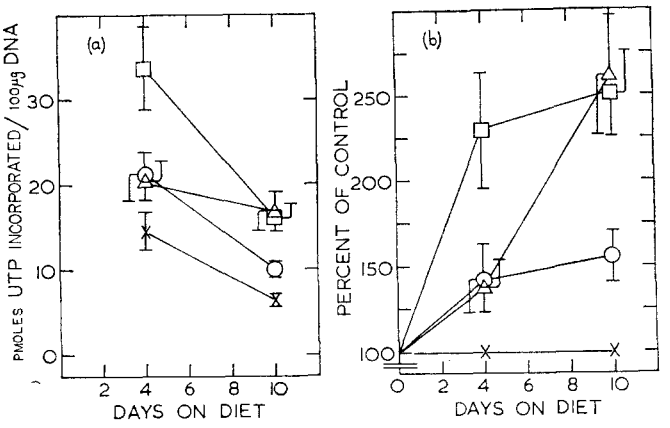


Fig. 3. Effect of pair-feeding a control diet × — × and diets containing CAP, △ — △, 3′MeDAB, ○ — ○ or 3′MeDAB plus CAP □ — □ to rats on ^{14}C–UTP incorporation (Mg^{2+}-stimulated, pH 8·5) into RNA in isolated liver nuclei. Incubations were performed for 6 min at 37°C. Each point represents the mean ± S.E. of duplicate estimations on nuclei from 4 or 5 rats; 3(a) shows absolute values and 3(b) results expressed as a percentage of the respective control values.

nuclear RNA synthesis [23–25]. Nuclei from rats which had received 3′MeDAB and CAP showed significantly greater incorporation than those from control rats at 4 ($P < 0·02$) and 10 ($P < 0·01$) days. At 10 days, the increases in incorporation in rats fed CAP ($P < 0·01$) and 3′MeDAB ($P < 0·02$) were also significant, but incorporation in nuclei from rats fed 3′MeDAB and CAP was significantly greater than in nuclei from rats fed 3′MeDAB ($P < 0·02$). The $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity (45 min incubation) was significantly increased relative to the control group in rats receiving both diets containing CAP for 4 days (Table 1). When incubations were performed for 15 min only, the differences between groups were not as apparent, but increases in incorporation rela-

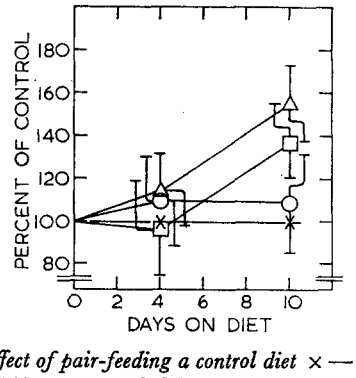


Fig. 4. Effect of pair-feeding a control diet × — × and diets containing CAP, △ — △, 3′MeDAB, ○ — ○ or 3′MeDAB plus CAP □ — □ to rats on ^{14}C–UTP incorporation (Mn^{2+}/$(NH_4)_2SO_4$-stimulated, pH 7·5) into RNA in isolated liver nuclei. Incubations were performed for 15 min at 37°C following a 15 min preincubation. Each point represents the mean ± S.E. of duplicate estimations on nuclei from 3 rats at 4 days and 4 or 5 rats at 10 days.

tive to control were once more observed in both groups of rats fed CAP-containing diets (Fig. 4); these were more pronounced at 10 days and were significant ($P < 0{\cdot}05$) at this time in the group fed CAP alone. Studies in which samples were incubated with or without α-amanitin revealed that the toxin inhibited 88·6% of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity but only 20·6% of the Mg^{2+}-stimulated activity (Fig. 5);

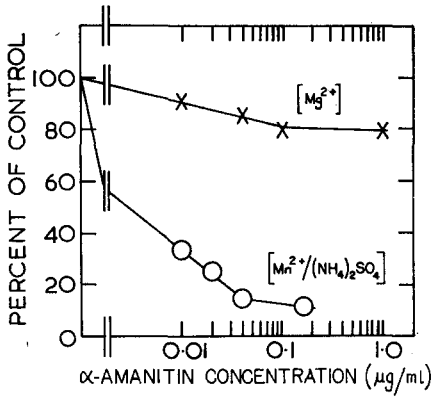


Fig. 5. Effect of α-amanitin on incorporation of ^{14}C-UTP into RNA in nuclei isolated from a pooled homogenate obtained from 2 control rats. Incubations were for 6 min at 37°C in the Mg^{2+}-stimulated reaction and for 15 min at 37°C following a 15 min preincubation in the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction. α-Amanitin was added at zero time in both reactions. Each point represents a single analysis on a separate aliquot of nuclear suspension.

these figures were of the same order in nuclear preparations from rats in each of the experimental groups and indicate that, while the greater part of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction is due to RNA polymerase II, this enzyme only participates to a minor extent in the Mg^{2+}-stimulated reaction.

The nuclear RNA/DNA ratio was significantly increased following 10 days administration of the CAP-containing diet (Table 2). Furthermore, the nuclear RNA/DNA ratio was greater in rats receiving both 3'MeDAB and CAP than in those receiving 3'MeDAB alone. Although the latter difference was not significant at either 4 or 10 days, if both groups of data were pooled prior to statistical analysis, a significant difference was demonstrable ($P < 0{\cdot}05$).

The % recovery of nuclei from the livers of rats in the various groups was determined by estimating the % recovery of homogenate DNA in the nuclear preparations (Table 3). The lower recovery observed in the treated groups may be a result of increased nuclear fragility in the larger livers of the treated rats or alternately might reflect differences in nuclear size, shape and density which could affect their sedimentation characteristics. The RNA/DNA ratios of the nuclei isolated in this experiment (Table 3) conformed to the pattern shown in Table 2, while the homogenate RNA/DNA ratios were similar to those reported earlier [2].

Morphological examination of the isolated nuclei from control rats and rats fed CAP revealed some agglutination. Counts were performed on nuclear suspensions where the degree of agglutination permitted, and the control values (mean ± 1 S.D.) were $36{\cdot}45 \pm 6{\cdot}44 \times 10^7$ nuclei/ml of nuclear suspension at 4 days (5 rats) and $42{\cdot}54 \pm 5{\cdot}66 \times 10^7$ nuclei/ml of nuclear suspension at 10 days (4 rats). The nuclear counts in the treated groups were not significantly different from the control group at 4 days, but at 10 days there were significantly less ($P < 0{\cdot}05$) nuclei/ml of nuclear suspension in preparations from rats fed 3'MeDAB and CAP ($33{\cdot}50 \pm 5{\cdot}50 \times 10^7$ nuclei/ml, 5 rats). As each nuclear preparation was obtained from 4 g of liver, this result means either that there were less nuclei/g of liver tissue in these rats as a consequence of an increase in the size of individual liver cells, or that loss of nuclei during isolation has been greater in this group.

Consideration was given to the possibility that the difference in incorporation of ^{14}C-UTP into nuclear RNA between the control and treated groups may be an artifact resulting from a spuriously low incorporation rate in the control group due to agglutination of the nuclei. Control nuclei were therefore isolated in 2·4 M sucrose containing various concentrations of Mg^{2+}, which has been reported to prevent agglutination [26]. Increasing concentrations of Mg^{2+} in the 2·4 M sucrose used for isolation of the nuclei resulted in less agglutination and an increase in the nuclear count (Table 4), presumably because aggregates of nuclei present in preparations isolated using 1 mM $MgCl_2$ were not counted. Nuclei isolated in 10 mM $MgCl_2$ showed maximal incorporation in the Mg^{2+}-stimulated reaction in agreement with previous findings using lysed nuclei [27] and those isolated in 3 mM $MgCl_2$ were most active in the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction, but neither of these increases was sufficiently great to account for the differences noted earlier between the control and treated groups (Figs. 3a, 3b and 4).

DISCUSSION

There is evidence that Mg^{2+}-stimulated nuclear RNA synthesis represents RNA synthesis in the nucleolus [6], which is the site of synthesis of ribosomal precursor RNA [28]. It was therefore interesting to note that the

Table 2. RNA/DNA ratios of nuclear preparations obtained from rat livers after pair-feeding rats for 4 and 10 days with diets containing CAP, 3'MeDAB or 3'MeDAB plus CAP

Group	4 Days on diet		10 Days on diet	
	RNA/DNA ratio	% Difference from control	RNA/DNA ratio	% Difference from control
Control	0·182 ± 0·020 (10)	—	0·161 ± 0·021 (5)	—
CAP	0·202 ± 0·025 (10)	+11·0	0·215 ± 0·034 (5)	+33·5*
3'MeDAB	0·171 ± 0·019 (9)	− 6·0	0·164 ± 0·022 (4)	+ 1·9
3'MeDAB plus CAP	0·195 ± 0·032 (10)	+ 7·1	0·178 ± 0·025 (5)	+10·6

The DNA and RNA contents were estimated on zero time samples from the Mg^{2+}-stimulated reaction as described in "Materials and Methods". Results are presented as the mean ± S.D. Numbers in parentheses indicate the number of experiments.

*Significantly different from control group as determined by Student's *t*-test ($P < 0·02$).

Table 3. % recovery of total homogenate DNA in isolated nuclear preparations and RNA/DNA ratios of nuclear preparations and whole liver homogenates following 10 days of pair-feeding rats with diets containing CAP, 3'MeDAB or 3'MeDAB plus CAP

Group	% Recovery of DNA	Nuclear preparation		Homogenate	
		RNA/DNA ratio	% Difference from control	RNA/DNA ratio	% Difference from control
Control	57·4	0·146	—	2·208	—
CAP	50·2	0·188	+28·8	2·394	+8·4
3'MeDAB	49·9	0·162	+11·0	2·051	−7·1
3'MeDAB plus CAP	46·2	0·174	+19.2	2·210	+0.1

The values shown represent the mean of triplicate estimations performed on one rat in each dietary group.

increases found in the present study in Mg^{2+}-stimulated RNA synthesis in rats fed diets containing either CAP or 3'MeDAB plus CAP for 4 or 10 days paralleled increases in total liver RNA [3], of which the major part is ribosomal RNA. Similarly, although there was only a slight increase in Mg^{2+}-stimulated nuclear RNA synthesis at 4 days in rats fed 3'MeDAB, there was a greater increase at 10 days, possibly accounting for the delayed increase in total liver RNA in these rats [3]. It is possible that the significant difference in Mg^{2+}-stimulated nuclear RNA synthesis between rats fed 3'MeDAB plus CAP and those fed 3'MeDAB alone might result in the difference in liver RNA/DNA ratio between these rats [2], as total liver DNA has been shown to increase at the same rate in each group [3]. The increases in Mg^{2+}-stimulated nuclear RNA synthesis following 10 days' administration of the various diets coincide with increases in microsomal protein synthesis [29], a finding which is in agreement with evidence that ribosomal RNA and protein synthesis are coordinated [30].

The increases that were noted in Mn^{2+}/$(NH_4)_2SO_4$-stimulated nuclear RNA synthesis

Table 4. Effect of varying the Mg^{2+} concentration in the nuclear isolation medium on the nuclear count, morphology and RNA synthetic activity of isolated rat liver nuclei

Mg^{2+} concentration in 2·4 M sucrose (mM)	Nuclear count/ml nuclear suspension $\times 10^{-7}$	Nuclear morphology	RNA synthetic activity	
			Mg^{2+}-stimulated pH 8·5	$Mn^{2+}/(NH_4)_2SO_4$-stimulated pH 7·5
1	30·6	A large number of nuclei appeared disrupted (~30%); contamination with what appear to be membrane fragments was moderate; agglutination was pronounced.	11·6	11·8
3	32·9	Nuclear disruption and agglutination was less; contamination was moderate. Nuclei appeared smaller than those isolated in 1mM $MgCl_2$.	12·4 (+7·4%)	14·5 (+23·0%)
5	34·3	Nuclei appeared similar to those isolated in 3mM $MgCl_2$; contamination was slight and agglutination was not apparent.	11·5 (−1·0%)	11·3 (−4·2%)
10	40·2	Nuclei appeared shrunken and very darkly staining; contamination was slight and there was no agglutination.	13·8 (+18·9%)	12·0 (+2·0%)

RNA synthetic activity is expressed as pmoles incorporated/100 μg DNA. Duplicate samples of nuclei from a control rat were incubated for 6 min at 37°C in the case of the Mg^{2+}-stimulated reaction and for 15 min after a 15-min preincubation at 37°C in the case of the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction. The % difference from the respective control samples (nuclei isolated in 1mM $MgCl_2$) are shown in parentheses.

in rats fed diets containing either CAP or 3'MeDAB plus CAP at 4 days were of interest, as they may be responsible for the increases noted in the Mg^{2+}-stimulated reaction. Recent evidence suggests that a protein with a rapid turnover is necessary for nucleolar RNA synthesis and that its messenger RNA has a very short half-life [31], a suggestion which is consistent with reports that a product of the nucleoplasmic RNA polymerase (which, like the $Mn^{2+}/(NH_4)_2SO_4$-stimulated reaction produces DNA-like RNA) may be necessary for increases in activity of the nucleolar enzyme [32–34].

There are many reports of the effects of various azo dyes on liver RNA synthesis, but most of these are not directly comparable with the present data as the dye was administered by different routes or incorporation of radioactive precursor was measured following administration *in vivo*. The increase in RNA synthesis in isolated liver nuclei following dietary administration of a carcinogenic azo dye noted in the present study is in agreement with other reports [35–38] in which liver RNA synthesis has been measured *in vitro* following the feeding of such dyes to rats for periods of from 1 to 3 weeks. The increase in the Mg^{2+}-stimulated reaction that was noted following the feeding of a diet containing 3'MeDAB for 10 days is in agreement with data of Akao *et al.* [35], but the decrease in the $Mn^{2+}/(NH_4)_2SO_4$-stimulated activity following dye administration reported by these workers [35] is in contrast with the present results. This effect could have been a consequence of a lower dietary intake in rats fed 3'MeDAB as compared with the control rats in their study, as no mention was made of pair-feeding.

The observation that both Mg^{2+}-stimulated and $Mn^{2+}/(NH_4)_2SO_4$-stimulated nuclear RNA synthesis were enhanced by dietary CAP is of interest as in earlier studies CAP was reported to inhibit RNA synthesis in rat liver cell suspensions [39]. This finding may indicate that a metabolite of CAP is responsible for the effects observed *in vivo*, that the concentration of CAP used in the *in vitro* studies far exceeded that present *in vivo* or that CAP affects metabolic conversion of RNA precursors.

The increase in nuclear RNA synthesis in rats protected by concurrent administration of CAP as compared with rats fed the carcinogen alone is noteworthy and may be implicated in the protective effect. Thus Akao *et al.* [35] reported a similar effect induced by nitrofuran admini-

stration, which also inhibits azo dye carcinogenesis in rat liver. Furthermore, the inhibitors 3-methylcholanthrene and *p*-hydroxypropiophenone have been shown to reverse alterations induced in the nucleolus by carcinogenic azo dyes [40, 41]. There is evidence that the synthesis of specific RNAs is necessary for transition from one phase of mitosis to the next [42], so the effects of CAP on RNA synthesis in the liver of 3'MeDAB-fed rats may be implicated in the restoration to normal of the phase distribution of the mitotic response in these animals [2]. Nevertheless, caution must be used in the interpretation of studies on nuclei isolated from rats which have been treated with exogenous compounds. There is evidence of an alteration in the population of rat liver nuclei within a few days of commencing azo dye administration [43, 44] and furthermore, different populations of liver nuclei have been shown to vary with regard to the extent of utilization of different RNA precursors administered *in vivo* [45] and the rate of RNA synthesis measured *in vitro* [46]; it is therefore possible that such factors could influence the results reported here. However, an earlier morphological study [47] revealed no differences in liver architecture following pair-feeding diets containing CAP, 3'MeDAB or both compounds for a period of 4 days, a time when there were significant differences in nuclear RNA synthesis between the control and CAP-protected rats.

The decrease in the liver nuclear RNA/DNA ratio following the dietary administration of carcinogenic azo dyes is in agreement with data of Sporn and Dingman [48]. As a decrease was not produced by the weakly carcinogenic 2-methyl-4-dimethylaminoazobenzene [48], the significant increase in this ratio in the nuclei of CAP-protected rats to a value approaching the control level might be relevant to the protective effect. If degradation of nuclear RNA during the Mg^{2+}-stimulated reaction *in vitro* is a guide to events taking place *in vivo*, there is evidence that degradation is less extensive in rats receiving both 3'MeDAB and CAP than in rats on the other diets; this, plus an increased rate of RNA synthesis, could account for the increase in the nuclear RNA/DNA ratio as compared with rats receiving 3'MeDAB.

Azo dye derivatives have been shown to react *in vivo* with DNA, RNA and protein [49] and it is possible that a resultant modification of either RNA polymerase or the chromatin template may alter the response of the cell to increased demands for RNA synthesis following sublethal cytoplasmic damage. Further studies are required to determine first whether the liver cell is incapable of a maximal response to damage during carcinogenesis, secondly whether the ability to respond to cellular injury is dependent on optimum levels of RNA synthesis and thirdly those aspects of RNA synthesis that are modified by the carcinogenic azo dyes.

REFERENCES

1. A. Lacassagne and L. Hurst, Action retardatrice du chloramphénicol sur le processus de cancérisation du foie du rat par le *p*-dimethylaminoazobenzène (DAB). *Bull. Ass. franç. Cancer* **54,** 405 (1967).
2. J. M. Blunck, Alteration of the RNA/DNA ratio of rat liver associated with the prevention of azo dye-induced carcinogenesis by dietary chloramphenicol. *Life Sci.* (II) **9,** 51 (1970).
3. J. M. Blunck, Inhibition by chloramphenicol of aminoazo dye carcinogenesis in rat liver: studies of biochemical changes in rat liver and protein binding of carcinogen. *Chem.-Biol. Interactions* **2,** 217 (1970).
4. A. Cooke, J. E. Kay and H. L. Cooper, Ribonucleic acid polymerase activity as a measure of ribonucleic acid synthesis. *Biochem. J.* **125,** 74P (1971).
5. C. C. Widnell and J. R. Tata, Studies on the stimulation by ammonium sulphate of the DNA-dependent RNA polymerase of isolated rat-liver nuclei. *Biochim. biophys. Acta* (*Amst.*) **123,** 478 (1966).
6. A. O. Pogo, V. C. Littau, V. G. Allfrey and A. E. Mirsky, Modification of ribonucleic acid synthesis in nuclei isolated from normal and regenerating liver: some effects of salt and specific divalent cations. *Proc. nat. Acad. Sci.* (*Wash.*) **57,** 743 (1967).
7. R. G. Roeder and W. J. Rutter, Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature* (*Lond.*) **224,** 234 (1969).
8. R. G. Roeder and W. J. Rutter, Specific nucleolar and nucleoplasmic RNA polymerases. *Proc. nat. Acad. Sci.* (*Wash.*) **65,** 675 (1970).
9. R. H. Reeder and R. G. Roeder, Ribosomal RNA synthesis in isolated nuclei. *J. molec. Biol.* **67,** 433 (1972).

10. S. T. Jacob, E. M. Sajdel and H. N. Munro, Presence of two RNA polymerase activities in isolated nucleoli. *Biochim. biophys. Acta (Amst.)* **157,** 421 (1968).
11. F. Yu and P. Feigelson, Studies on the role of ammonium sulfate in nuclear transcription *in vitro*. *Biochim. biophys. Acta (Amst.)* **272,** 119 (1972).
12. T. J. Lindell, F. Weinberg, P. W. Morris, R. G. Roeder and W. J. Rutter, Specific inhibition of nuclear RNA polymerase II by α-amanitin. *Science N.Y.* **170,** 447 (1970).
13. J. E. Giese, J. A. Miller and C. A. Baumann, The carcinogenicity of *m'*-methyl-*p*-dimethylaminoazobenzene and of *p*-monomethylaminoazobenzene. *Cancer Res.* **5,** 337 (1945).
14. C. C. Widnell and J. R. Tata, A procedure for the isolation of enzymically active rat-liver nuclei. *Biochem. J.* **92,** 313 (1964).
15. G. Blobel and V. R. Potter, Distribution of radioactivity between the acid-soluble pool and the pools of RNA in the nuclear, nonsedimentable and ribosome fractions of rat liver after a single injection of labelled orotic acid. *Biochim. biophys. Acta (Amst.)* **166,** 48 (1968).
16. H. N. Munro and A. Fleck, Recent developments in the measurement of nucleic acids in biological materials. A supplementary review. *Analyst* **91,** 78 (1966).
17. H. N. Munro and A. Fleck, The determination of nucleic acids. *Methods of Biochemical Analysis* **14,** 113 (1966).
18. A. I. Meisler and B. E. Tropp, Studies on ribonucleic acid synthesis in nuclei isolated from rat liver. *Biochim. biophys. Acta (Amst.)* **174,** 476 (1969).
19. G. S. Edwards and G. N. Wogan, Ribonuclease activity in liver nuclei of aflatoxin-treated rats. *Life Sci.* (II) **11,** 685 (1972).
20. J. D. Johnson, B. A. Jant, S. Kaufman and L. Sokoloff, Effects of ionic strength on the RNA polymerase activities of isolated nuclei and nucleoli of rat liver. *Arch. Biochem.* **142,** 489 (1971).
21. F. Novello and F. Stirpe, Experimental conditions affecting ribonucleic acid polymerase in isolated rat liver nuclei. *Biochem. J.* **112,** 721 (1969).
22. P. Chambon, M. Ramuz, P. Mandel and J. Doly, The influence of ionic strength and a polyanion on transcription *in vitro*. I Stimulation of the aggregate RNA polymerase from rat liver nuclei. *Biochim. biophys. Acta (Amst.)* **157,** 504 (1968).
23. D. Rickwood and H. Klemperer, Decreased RNA synthesis in isolated rat liver nuclei during starvation. *Biochem. J.* **120,** 381 (1970).
24. A. Henderson, The effect of feeding with a tryptophan-free amino acid mixture on rat liver magnesium ion-activated DNA-dependent RNA polymerase. *Biochem. J.* **120,** 205 (1970).
25. T. Onishi, Studies on the mechanism of decrease in the RNA content in liver cells of fasted rats. *J. Biochem. (Tokyo)* **67,** 577 (1970).
26. J. St L. Philpot and J. E. Stanier, The choice of the suspension medium for rat-liver-cell nuclei. *Biochem. J.* **63,** 214 (1956).
27. G. Capobianco and A. Vescia, Some factors affecting DNA-dependent RNA polymerase solubilization from rat liver nuclei. *Ital. J. Biochem.* **19,** 217 (1970).
28. R. P. Perry, The cellular sites of synthesis of ribosomal and 4 S RNA. *Proc. nat. Acad. Sci. (Wash.)* **48,** 2179 (1962).
29. J. M. Blunck and N. P. Madsen, *In vitro* incorporation of [1-^{14}C] leucine into rat liver microsomal protein after dietary supplementation with 3'methyl-4-dimethylaminoazobenzene, chloramphenicol or both compounds. *Chem.-Biol. Interactions* **4,** 209 (1972).
30. K. Tsurugi, T. Morita and K. Ogata, Effects of the inhibition of ribosomal-RNA synthesis on the synthesis of ribosomal structural proteins in regenerating rat liver. *Europ. J. Biochem.* **29,** 585 (1972).
31. F. Yu and P. Feigelson, The rapid turnover of RNA polymerase of rat liver nucleolus, and of its messenger RNA. *Proc. nat. Acad. Sci. (Wash.)* **69,** 2833 (1972).
32. S. Jacob, W. Muecke, E. Sajdel and H. Munro, Evidence for extranucleolar control of RNA synthesis in the nucleolus. *Biochem. biophys. Res. Commun.* **40,** 334 (1970).
33. C. Raynaud-Jammet, M. G. Catelli and E. E. Baulieu, Inhibition by α-amanitin of the oestradiol-induced increase in α-amanitin insensitive RNA polymerase in immature rat uterus. *FEBS Lett.* **22,** 93 (1972).

34. W. SCHMID and C. E. SEKERIS, Sequential stimulation of extranucleolar and nucleolar RNA synthesis in rat liver by cortisol. *FEBS Lett.* **26,** 109 (1972).
35. M. AKAO, K. KURODA and K. MIYAKI, Selective inhibition of RNA polymerase activity in rat liver nuclei by 4-(dimethylamino)-azobenzene, and effect of nitrofurans on liver RNA metabolism associated with prevention of carcinogenesis. *Gann* **63,** 1 (1972).
36. J. S. WORTHAM, R. G. WILSON, H. L. FALK and H. V. GELBOIN, The effect of a series of aminoazo dyes, aflatoxin and certain liver microsomal enzyme inducers on rat liver RNA polymerase. *Proc. Am. Ass. Cancer Res.* **8,** 74 (1967).
37. K. R. REES and G. F. ROWLAND, The metabolism of isolated rat-liver nuclei during chemical carcinogenesis. Thioacetamide and *p*-dimethylaminoazobenzene (butter yellow). *Biochem. J.* **80,** 428 (1961).
38. E. L. R. GODWIN, K. R. REES and J. S. VARCOE, Nuclear RNA synthesis in rat liver during the early stages of chemical carcinogenesis. *Brit. J. Cancer* **21,** 166 (1967).
39. S. T. JACOB and P. M. BHARGAVA, Effect of chloramphenicol on ribonucleic acid synthesis in liver cells in suspension. *Biochem. J.* **97,** 67 (1965).
40. M. O'HEGARTY and J. HARMAN, The inhibitory effect of 3-methylcholanthrene on nucleolar alterations induced in rat liver by 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.* **29,** 521 (1969).
41. N. UNAKAR and M. COLE, JR., Nucleolar alterations of hepatocytes during carcinogenesis. *J. Cell Biol.* **43,** 149a (1969).
42. A. GONZÁLEZ-FERNÁNDEZ, M. FERNÁNDEZ-GÓMEZ, J. STOCKERT and J. LÓPEZ-SÁEZ, Effect produced by inhibitors of RNA synthesis on mitosis. *Exp. Cell Res.* **60,** 320 (1970).
43. T. W. SNEIDER, D. E. BUSHNELL and V. R. POTTER, The distribution and synthesis of DNA in two classes of rat liver nuclei during azo dye-induced hepatocarcinogenesis. *Cancer Res.* **30,** 1867 (1970).
44. H. M. RABES, R. HARTENSTEIN and W. RINGELMANN, DNA synthesis in rat liver during early stages of azo dye-induced hepatocarcinogenesis. *Cancer Res.* **32,** 83 (1972).
45. D. E. BUSHNELL, E. D. WHITTLE and V. R. POTTER, Differential utilization of pyrimidines for RNA synthesis in two classes of rat liver nuclei. *Biochim. biophys. Acta (Amst.)* **179,** 497 (1969).
46. I. R. JOHNSTONE, A. P. MATHIAS, F. PENNINGTON and D. RIDGE, The fractionation of nuclei from mammalian cells by zonal centrifugation. *Biochem. J.* **109,** 127 (1968).
47. J. M. BLUNCK, Inhibition by chloramphenicol of aminoazo dye carcinogenesis in rat liver: morphological studies. *Pathology* **3,** 99 (1971).
48. M. B. SPORN and C. W. DINGMAN, Studies on chromatin. II Effects of carcinogens and hormones on rat liver chromatin. *Cancer Res.* **26,** 2488 (1966).
49. E. C. MILLER and J. A. MILLER, Mechanisms of chemical carcinogenesis: nature of proximate carcinogens and interactions with macromolecules. *Pharmacol. Rev.* **18,** 805 (1966).

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Studies on Steroid Sulphation and Binding by Human Malignant Tumours

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Abstract—*Sulphation of dehydroepiandrosterone and oestradiol-17β* in vitro *and oestrogen binding* in vivo *and* in vitro *by human malignant breast tumours have been studied. Although both parameters have been claimed to be related to tumour response following endocrine ablation, no correlation between sulphation and binding could be demonstrated. Enzymes effecting steroid sulphation are present in malignant tumours of tissues other than breast.*

INTRODUCTION

ENDOCRINE therapy of advanced breast cancer fails to achieve a successful response in the majority of cases and a search for biochemical "predictors" of response has been in progress for the last two decades. Two properties of human breast tumour tissue—its ability to effect steroid sulphation [1] or to bind oestradiol [2]—have been claimed to be related to the patient's clinical response after endocrine ablation.

That both primary and metastatic mammary carcinoma tissues contain steroid sulphokinases was first shown by Adams [3]. Dao and Libby [4] confirmed the formation of steroid sulphates by preparations of metastatic tumour tissue and noted that the yields of oestradiol sulphate (E_2S) were relatively variable compared to those of other steroid sulphates. Using dehydroepiandrosterone sulphate (DHAS) formation as a reference point, they estimated relative yields of E_2S and suggested [1, 5] that either an absence of steroid conjugating activity or high rates of oestradiol (E_2) conjugation relative to those for dehydroepiandrosterone (DHA) were associated with poor responses of tumours to adrenalectomy. Lack of steroid sulphating activity in tumour preparations was shown to be due to the absence of sulphate activating enzymes rather than steroid sulphokinases [6].

The ability of a human malignant breast tumour to concentrate E_2 *in vivo* was first demonstrated by Pearlman and co-workers [7]. Confirming this work, Braunsberg, Irvine and James [8] were able to show that some, but not all, breast cancers contained relatively high concentrations of oestrogen and that normal breast and benign breast lesions did not appear to take up the hormone or its metabolites. One benign tumour, an intraduct papilloma, was later shown to do so [9]. When tumour oestrogen concentrations were high, this was largely due to oestradiol itself [10]. *In vitro* estimations of oestradiol binding by human breast tumour slices [11–13] or cell-free preparations [2, 11, 14–16] confirmed that only a proportion of tumours exhibit an affinity for oestradiol and Jensen *et al.* [11] have presented data which suggest that absence of binding components may be associated with lack of response of the tumour to endocrine ablation.

If two apparently diverse properties, oestradiol conjugation and binding, were related to tumour response, they might be expected to be correlated, and the present work was undertaken to test this hypothesis. Steroid sulphation

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in vitro and E_2 binding either *in vivo* or *in vitro* were studied in parallel in a series of human breast tumours. In addition, steroid sulphating activities were investigated in normal breast tissue, benign breast lesions and malignant tumours of tissues other than breast for comparison with breast carcinoma tissue.

MATERIAL AND METHODS

Tris (hydroxymethyl) aminomethane ("Trizma base"), adenosine 5′-triphosphate (ATP, Sigma grade, 99–100%, low calcium content, from equine muscle) and activated charcoal (Norit A) were from Sigma London Chemical Co. Ltd. Benzene, toluene, petroleum ether (boiling range 60–80°), methanol, ethyl acetate, chloroform, dichloromethane, chromium trioxide and sulphuric acid (all Analar), cyclohexane and fluorescein (reagent grade), 2-methoxyethanol and non-radioactive oestradiol-17β were from BDH Chemicals Ltd., Poole, Dorset. Naphthalene was from May and Baker, Dagenham, Essex.

Non-radioactive dehydroepiandrosterone was from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Oestradiol-17β-4-^{14}C (51·4 mCi/mmole), oestradiol-17β-6,7-^{3}H (40-55·6 Ci/mmole), oestradiol-17β-2,4,6,7-^{3}H (100 Ci/mmole) and sodium-^{35}S-sulphate (initial specific activities 18·1–55·2 mCi/mmole) were from the Radiochemical Centre, Amersham, Bucks. The purity of oestradiol-17β-6,7-^{3}H used for infusions was checked by addition of purified oestradiol-17β-4-^{14}C, thin layer chromatography, oxidation to oestrone and rechromatography using the ^{14}C/^{3}H ratio as criterion of purity [10]. Storage at −15° in benzene at concentrations not exceeding 10 μCi/ml was satisfactory for at least two months. Oestradiol-17β-2,4,6,7-^{3}H used for *in vitro* assay of E_2 binding proved to be more labile and was purified immediately before use on columns of Sephadex LH20 (Pharmacia, Uppsala, Sweden) [17]. Fractions of 2 ml were collected and the material contained in the fraction with the highest tritium concentration was used.

Aqueous samples and steroid conjugates were counted in 15 ml BBOT scintillator: 6g BBOT (2,5-bis-[2-(5-*t*-butyl-benzoxaloyl)] thiophene, Ciba Ltd., Horsham, Sussex), 80 g naphthalene, 400 ml methoxyethanol, and 600 ml toluene. Free steroid extracts were counted in 15 ml toluene scintillator: 3g *p*-terphenyl and 40 mg dimethyl-POPOP (both from Nuclear Enterprises, Edinburgh) per litre toluene.

Radioactive solutions were counted in a Beckman (Model LS 233, Beckman Instruments, Glenrothes) or a Tracerlab (Corumatic 200, Baird Atomic Ltd., Braintree, Essex) liquid scintillation counter. For counting ^{35}S the Beckman isoset for ^{14}C (single isotope) was used, giving an efficiency of 75%, and samples were counted twice for 50 min. The extracts from infusion studies were counted under double isotope conditions for ^{14}C and ^{3}H and those from *in vitro* oestradiol binding assays under single isotope conditions for ^{3}H. A minimum of 8000 counts (theoretical coefficient of variation 1·2%) was accumulated for most samples, with the exception of a few which gave low recoveries of ^{14}C-oestradiol in the *in vivo* infusion studies. For these samples at least 5000 counts (theoretical coefficient of variation 1·4%) were accumulated.

Tissues obtained at operation were immediately placed on ice and brought to the laboratory within 3 hr. Preparations for *in vitro* binding assay and for sulphation studies were made immediately. Tumour samples were placed on a tile on a refrigerated tray and trimmed as free as possible of adipose tissue, chopped finely and carefully homogenised in a stainless steel homogeniser (Silverson Machines Ltd., London) with the glass tube immersed in ice. Tissues collected from patients infused with labelled oestradiol were sampled and analysed for steroid sulphation activity and the remainder stored at −15° while awaiting determination of free steroid radioactivity. Malignancy was confirmed by histological examination.

Estimation of steroid sulphation

Supernatants (12,000g) were prepared according to the method used by Dao and Libby [4] except that 1μCi (20–60 nmoles) of $Na_2{}^{35}SO_4$ was used, and incubation continued for 90 min. These modifications resulted in slightly higher yields of labelled sulphates. Blanks consisted of the same constituents as test samples, but tissue supernatant preparations were added after incubation and cooling. These blanks gave the same value as blank incubations with tissue supernatants but without added steroids, indicating that no sulphates formed from endogenous tissue components were extracted into the steroid sulphate fraction. All assays of tissue supernatant preparations were carried out in duplicate or triplicate.

Estimations of protein contents of tissue samples were carried out as described by Lowry *et al.* [18].

Estimation of oestrogen binding

Oestrogen binding by breast tumour tissues

in vivo was estimated by constant infusion of ^{3}H-oestradiol as described previously [19], using Method II for analysing tissues and Method III for plasma samples. All analyses were carried out in duplicate, triplicate or (rarely) quadruplicate and widely separate areas of tumour were sampled whenever possible.

Oestradiol binding *in vitro* was determined by the method of Feherty *et al.* [14], which estimates the concentration (P_0) of unoccupied high affinity binding sites (oestradiol "receptors") and the dissociation constant (K_d) of the oestradiol "receptor" complex at equilibrium at 30°. This technique has a sensitivity of $0{\cdot}1 \times 10^{-15}$ moles/mg. wet weight [14]. All assays on tumour supernatant preparations were carried out in duplicate.

Estimation of precision

Variances were calculated from replicate determinations using the formula

$$V = \frac{\sum_{i=1}^{n}\left(\Sigma x^2 - \frac{(\Sigma x)^2}{n_i}\right)}{\Sigma n_i - N} \qquad (1)$$

where x are the n_i values for the ith sample and N is the number of samples analysed. When assays are carried out in duplicate, this equation reduces to

$$V = \frac{\Sigma d^2}{2N'} \qquad (2)$$

where d are differences between duplicates and N' is the number of duplicate analyses [20].

For individual tumours the variances of the sulphation index (S.I., ratio of yield of DHAS to that of E_2S [1]) and of the relative oestrogen concentration (R.O.C., ratio of radioactivity per unit weight of tumour to that per unit volume of plasma [8] following infusion of tritium-labelled oestradiol), were estimated from [21]:

$$V_R = \frac{V_A}{B^2} + \frac{A^2}{B^4} V_B \qquad (3)$$

where V_R is the variance of the estimated ratio A/B, V_A and V_B are the variances, calculated from Equations (1) or (2) and A and B are the expected values (means) of the two parameters, which, for this purpose, can be considered statistically independent. The ratios DHAS/E_2S were calculated from the net countrates (sample minus blank). Therefore the variance of the sulphation index was estimated by calculating V_A and V_B as sums of the variances for samples and blanks. For the relative oestrogen concentrations V_A and V_B were calculated directly from the variances obtained from Equations (1) or (2) using replicate results for tissue and plasma in terms of counts/min per unit weight or volume.

RESULTS

When sulphation was measurable in preparations of malignant breast tumours, yields of DHAS and E_2S estimated for 16 tumours ranged from 10 to 170 and from 8 to 170 pmoles per mg protein per hr, respectively, and sulphation indices (S.I., ratio of yield of DHAS to that of E_2S) estimated in 24 tumours varied between 0·3 and 2·3.

The variance for blanks used in the estimations of sulphation was calculated from a total (Σn_i) of 29 determinations prepared in 13 experiments. This was added to the variances for DHAS and E_2S with Σn_i 35 and 42 and N equal to 15 and 16, respectively. The variance for the sulphation index was estimated from Equation (3), yielding a standard deviation of 0·339. The mean value of S.I. was 1·2, and the coefficient of variation was 28·2%. Since all results in the present paper are based on duplicate or triplicate determinations, this coefficient would be reduced to about 20%.

In the estimation of tissue radioactivity following infusion of tritium-labelled oestradiol, the variances (calculated from equation (i)) were 9486 with a mean of 479 (N = 46, range = 162–949) at levels less than 1000 counts/min per g, and 104488 with a mean of 2142 (N = 15, range = 1152–7749) at levels greater than 1000 counts/min per g. The corresponding coefficients of variation were 20·3 and 15·1%. The estimated variance for plasma samples was 185 with a mean of 278 counts/min per ml (N = 61; range 103–472) and the coefficient of variation was 4·9%. Using Equation (3) the estimated variances for tissue/plasma ratios were 0·130 at the lower tissue count rates and 1·494 at the higher level and the corresponding mean ratios were 1·94 and 6·86, respectively. Hence the coefficients of variation were 18·6 and 17·8% at the two levels, and since all estimates were based on means of at least two replicate assays, the coefficient of variation was reduced to about 12%.

It was not always possible to complete estimations of sulphation on the day the tumours were removed. However, tumour tissue could not be frozen prior to preparation of supernatants, since it was found that this led to complete lost of sulphation activity. On the other hand, moss freshly prepared tissue supernatants do not

appear to deteriorate markedly on freezing [22] and Table 1 shows the effect of storage at −15° on sulphation indices (S.I.) for three tumours. It would appear that in our hands no significant change was caused by short-term storage. Nevertheless, estimations of sulphation were carried out on fresh preparations whenever possible and where storage was unavoidable, this is indicated in Tables 2–4.

Table 1. *Effect of storage of supernatant preparations at* −15° *on sulphation index (S.I.)*

Tissue	No. of days stored	S.I.
Ca Breast	0	0·8
	1	0·9
Ca Breast	0	0·9
	1	1·2
Ca Ovary	1	0·8
	4	0·7

Table 2. *Sulphation index (S.I.) and relative oestrogen concentration (R.O.C.)*

Patient No.	Age (yr)	Storage (Days)[a]	S.I.[b]	R.O.C.[b]
1[c]	74	1	2·0	6·6
			—	10·0
2	45	3	1·1	0·7
3	54	1	1·0	3·5
4	41	1	1·0	1·4
5	40	3	1·0	0·8
			—	0·9[d]
6	66	5	0·8	3·5
7	55	5	0·7	0·7
			—	1·2[d]
8[e]	84	2	0·6	1·9
			N.D.	3·3
9	75	1	0·5	3·7
10[f]	68	0	0·3[d]	5·6[d]
11	57	1	(+)	1·2
12	65	1	(+)	9·9
13	64	0	N.D.	1·0
14	51	1	N.D.	1·0
15[e]	63	2	N.D.	1·6
		2	(+)	—
16	61	1	N.D.	3·2
17	69	0	N.D.	4·0
18	69	1	N.D.	4·7

(a) Time of storage at −15° of supernatant used for estimation of S.I.
(b) (+) detected at the limit of sensitivity;
N.D. no sulphation (or binding) detected; — not analysed
(c) Tumours in right and left breasts
(d) Metastasis (lymph node)
(e) Two separate tumours in same breast
(f) Patient on treatment with Decadurabolin

Table 3. *Sulphation index (S.I.) and concentration of oestradiol binding sites(Po)*

Patient No.	Age (yr)	Storage (Days)[a]	S.I.[b]	P_0[b]
19	57	0	2·3	1·1
20[c]	46	1	1·6	6·4
		1	1·8	4·2
21	75	1	1·3	4·3
2	45	3	1·1	N.D.
22	43	1	1·0	N.D.
5	40	3	1·0	N.D.
23	39	0	0·9	N.D.
24	61	1	0·8	N.D.
25	49	1	0·8	4·9
26	51	0	0·8[d]	N.D.[d]
27	53	1	0·7[e]	(+)[e]
28	65	2	0·6	11·7
29	59	0	N.D.	(+)
30	40	0	N.D.	N.D.
31	80	0	N.D.	1·8
32	58	0	N.D.	1·3
33	66	0	N.D.	6·4
34	59	0	N.D.	0·6
35	58	0	N.D.	1·5

(a) Time of storage at −15° of supernatant used for estimation of S.I.
(b) (+) sulphation (or binding) detected at the limit of sensitivity;
N.D. no sulphation (or binding) detected
(c) Tumour metastases from right and left ovaries
(d) Metastasis from ovary
(e) Metastasis (lymph node)

Sulphation activity was detected in 21 out of a total of 33 primary malignant breast tumours and in all five metastatic tumours examined. The primary tumour of patient 26 (Table 3) did not form steroid sulphates. Three lymph nodes removed from one patient and containing metastatic tumour tissue all effected sulphation. In two patients (Nos. 8 and 15, Table 2) two discrete tumours were found in the same breast. In both cases sulphation activity was detected in one and absent in the other tumour.

No sulphation activity was found in normal breast tissue (5 patients), fibroadenosis tissue (2 patients), fibroadenoma (2 patients), cysts removed from breasts (2 patients), and a sample of male gynaecomastia tissue. No activity was found in a cyst removed from the breast of a patient whose malignant breast tumour was active.

Table 2 shows a comparison of sulphation activity with *in vivo* estimates of relative oestrogen concentrations (R.O.C.) for 19 tumours. There was no evidence of correlation. Table 3 shows a comparison of sulphation activity and concentration of unoccupied high affinity oestradiol binding sites for 20 tumours, 2 of

which had also been examined by the infusion procedure. There was no evidence of correlation of the two properties.

Table 4 shows the results of sulphation studies on malignant tumours other than breast. Most of the tumours examined were active and sulphation indices varied from 0·3 to 3·5. A piece of normal colon yielded a sulphation index of 1·8.

DISCUSSION

The method for estimating steroid sulphation [4] used in the present work depends on the activity of a large number of enzymes in the *in vitro* system: those yielding and controlling the supply of active sulphate and the steroid sulphokinases and sulphatases. When steroid sulphates are detected, the amounts of PAPS (adenosine-3′-phosphate-5′-phosphosulphate) and sulphokinase activity would seem to be in excess of any sulphatase activity present. On the other hand, the absence of any detectable steroid sulphates may be due to inadequate amounts of PAPS or steroid sulphokinases, or to excess of sulphatases. Libby and Dao [6] have suggested that "inactive" breast tumour preparations can form steroid sulphates when sulphate activating enzymes are added. However, even after addition of these enzymes they found only very low yields of both DHAS and E_2S in 3 of 13 preparations studied. It would appear, therefore, that some tumours contain only traces of steroid sulphokinases or an excess of sulphatase activity. Alternatively, sulphokinase inhibitors may be responsible for low yields of steroid sulphates in some cases.

The conditions originally used by Dao and Libby [4] were adopted—with only slight modifications—in the present work, since their results appeared to be related to the progression of breast tumours [1, 5]. The yields of steroid sulphates and the range of sulphation indices found were of the same order as those described by Dao and Libby [1, 4, 5]. However, because of the poor precision for sulphation indices, division into equal to, greater than, or less than 1 [1] or any interpretation of their numerical values presents some problems. Dao and Libby [1, 5] have made no attempt to assess precision, but this is of some importance since they relate numerical values of the index to tumour response to adrenalectomy.

In a larger series of tumours than those included in the present work, detectable sulphation activity was present in 51% of 41 primary malignant breast tumours examined. This proportion is somewhat lower than that of 66% found by Dao and Libby [5] and could be due to the lower specific activity of $Na_2{}^{35}SO_4$ used here. It is of interest that Leung *et al.* [22] reported varying degrees of sulphation by all primary and metastatic malignant and by some benign tumours studied. However, in a later publication [24] the incidence of active primary tumours was 65 and 69% for DHA and E_2 sulphation respectively.

Table 4. Sulphation Index (S.I.) of Malignant tumours

Patient No.	Age (yr)	Storage (Days)[a]	Tumour	S.I.[b]
36	54	4	Adenocarcinoma of uterus	(+)
37	64	1	Adenocarcinoma of uterus	N.D.
38	67	1	Adenocarcinoma of uterus	1·7
39	70	1	Adenocarcinoma of uterus	0·6
40	68	1	Epidermoid carcinoma of uterus	0·8
41	89	1	Epidermoid carcinoma of uterus	0·3
42	51	1	Uterine sarcoma	N.D.
43	75	0	Adenocarcinoma of ovary	0·8
37	64	1	Ovarian thecoma	0·5
44	77	4	Squamous cell carcinoma of vulva (metastasis)	1·0
45	33	3	Carcinoma of testis	N.D.
46	33	0	Teratoma of testis	0·9
47	41	2	Hodgkin's disease (lymph node)	0·3
48	51	1	Adenocarcinoma of colon	3·5
49	77	0	Carcinoma of colon	0·9
50	62	3	Carcinoma of lung	(+)

(a) Time of storage at −15°.
(b) (+) sulphation detected at the limit of sensitivity; N.D. no sulphation detected.

In a later series of tumours [24] the incidence of active primary tumours was 65 and 69% for DHA and E_2 sulphation respectively.

Eleven of 22 tumours (50%) showed relatively high oestrogen uptakes (R.O.C. > 3) following infusion of tritium-labelled E_2 and 13 out of 20 tumours studied (65%) bound oestradiol *in vitro*, as judged by the method of Feherty *et al.* [14] who found binding in 37 out of 53 tumours (70%). In the studies of Jensen *et al.* [19] involving tissue slices or extracts 56% of 84 primary tumours bound oestradiol. Using tissue extracts Hähnel *et al.* [15] reported binding in 46% of 35 breast tumours, Korenman and Dukes [16] found 33% of 15 tumours and Maass *et al.* [23] 61% of 18 tumours. Using tissue slices, Sander [13] demonstrated oestradiol uptake in 9 out of 25 tumours (36%), Johansson *et al.* [12] found binding in 14 out of 31 tumours (45%) and Maass *et al.* [23] reported a proportion of 41% for 194 tumours. These proportions are remarkably similar in view of the different techniques adopted, though that found by Feherty *et al.* [14] was markedly higher than the others.

Although Dao and Libby [1] found steroid sulphation activity in 3 of 10 samples of normal breast tissue, none of the 5 samples examined in the present study produced steroid sulphates. None of the various benign breast lesions analysed showed activity. Similarly, Adams [3] could not detect DHAS formation in a cystic mastopathy specimen from a pre-menopausal woman.

The differences in sulphation activity between a primary tumour and its metastasis (patient 26) or between discrete tumours removed from 2 patients (Nos. 8 and 15) are of interest. When sulphation was effected in these cases, the sulphation indices were low.

The absence of any correlation between steroid sulphating activity and oestrogen binding, as estimated by two different techniques, is disappointing. The most clear-cut relationships to response after endocrine ablation are those between failure to regress and absence of steroid sulphation [1, 5] on the one hand or of oestradiol binding [2, 11] on the other. One would, therefore, expect negative findings for both parameters to occur in the same tumour. Of 6 tumours which failed to effect sulphation (patients 13–18, Table 2), 3 had low, 1 moderate and 2 high oestrogen uptakes in the *in vivo* infusion procedure. Of a further 7 tumours which lacked sulphation activity (patients 29 to 35, Table 3) 2 had little or no affinity for oestradiol and 5 bound this steroid, one with a high concentration of binding sites. For tumours which exhibited steroid sulphating activity, high or low oestrogen binding was found both by the infusion procedure or by the method of Feherty *et al.* [14]. The poor precision of the parameters estimated makes any quantitative interpretation of such a small number of comparisons difficult, but it would seem that the failure of a tumour to regress following endocrine ablation may be associated with a positive value for one and a negative finding for the other of the two biochemical parameters.

Our data do not entirely support those of Leung *et al.* [24], who reported that of 26 primary tumours studied, 13 both bound oestradiol and effected steroid sulphation. On the other hand, they were also unable to demonstrate any correlation between the two properties for tumours which contained little or no oestradiol receptor.

Apart from liver, normal human tissues have been shown to produce steroid sulphates *in vitro*. Thus adrenal [25–27], testis [28, 29] and jejunum [27] have been shown to contain steroid sulphokinases. The presence of sulphating activity in ovaries seems to be difficult to demonstrate [30, 31] because of the low levels present [3, 27, 32], but further evidence has been obtained recently [24, 33]. It is possible that the formation of DHAS and E_2S by preparations of ovarian metastases (patients 20 and 26, Table 3) could be explained by contamination with surrounding ovarian tissue.

Benign and malignant tumours of the adrenal [34–37] and a thyroid adenoma [27] have been shown to effect steroid sulphation. It seemed of interest to establish whether steroid and particularly oestradiol sulphating activity was commonly present in tumour tissue. The results in Table 4 show that a variety of malignant tumours formed measurable amounts of both DHAS and E_2S. However, the conditions for sulphation may not have been optimal; for example pH optima can vary for different tissues [27]. Nevertheless, it can be concluded that neither the ability to form DHAS and E_2S nor the variations in relative yields of these conjugates are characteristic for breast tumours, or even for hormone-dependent tissues.

For the management of patients with advanced carcinoma of the breast a reliable predictor of response is needed. If this were related to properties of the tumour tissue, then analysis of the primary tumour or of accessible metastases would be essential. Results of follow-ups of patients whose primary tumours have been examined for sulphation [5, 24] or oestrogen binding [11, 23, 24, 38] are still too few to assess their usefulness. It is possible that no single parameter will prove reliable and that a combination of several properties of the tumour,

as well as knowledge of the patient's pattern of steroid metabolism, is needed in the choice of treatment. Ultimately, only a full understanding of the effects of steroid therapy or endocrine ablation on tumour growth processes will provide a rationale for therapy.

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REFERENCES

1. T. L. Dao and P. R. Libby, Conjugation of steroid hormones by breast cancer tissue and selection of patients for adrenalectomy. *Surgery* **66,** 162 (1969).
2. E. V. Jensen, The pattern of hormone–receptor interaction. In: *Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer*, p. 151. Edited by K. Griffiths and C. G. Pierrepoint. Alpha Omega Alpha, Cardiff (1970).
3. J. B. Adams, Enzymic synthesis of steroid sulphates II. Presence of steroid sulphokinase in human mammary carcinoma extracts. *J. clin. Endocr.* **24,** 988 (1964).
4. T. L. Dao and P. R. Libby, Conjugation of steroid hormones by normal and neoplastic tissues. *J. clin. Endocr.* **28,** 1431 (1968).
5. T. L. Dao and P. R. Libby, Enzymic synthesis of steroid sulphate by mammary cancer and its clinical implications. *Natl. Cancer Inst. Monogr.* **34,** 205 (1971).
6. P. R. Libby and T. L. Dao, Lack of sulphate-activating enzymes in human breast tumours. *Proc. Soc. exp. Biol. (N.Y.)* **133,** 1409 (1970).
7. W. H. Pearlman, R. de Hertogh, K. R. Laumas, J. A. Brueggemann and M. R. J. Pearlman, Metabolism and tissue uptake of steroid sex hormones in patients with advanced carcinoma of the breast and in normal rats. In *Steroid Dynamics*, p. 159. Edited by G. Pincus, T. Nakao and J. F. Tait. Academic Press, New York (1966).
8. H. Braunsberg, W. T. Irvine and V. H. T. James, A comparison of steroid hormone concentrations in human tissues including breast cancer. *Brit. J. Cancer* **21,** 714 (1967).
9. H. Braunsberg, A. E. Carter, W. T. Irvine, F. James and V. H. T. James, Oestrogens in breast tumours. *Natl. Cancer Inst. Monogr.* **34,** 71 (1971).
10. F. James, H. Braunsberg, W. T. Irvine and V. H. T. James, Radioactivity in human breast tumours after infusion of ^{3}H-oestradiol: effect of androgen treatment. *Steroids* **15,** 669 (1970).
11. E. V. Jensen, G. E. Block, S. Smith, K. Kyser and E. R. de Sombre, Oestrogen receptors and breast cancer response to adrenalectomy. *Natl. Cancer Inst. Monogr.* **34,** 55 (1971).
12. H. Johansson, L. Terenius and L. Thorén, The binding of oestradiol-17β to human breast cancer and other tissues *in vitro*. *Cancer Res.* **30,** 692 (1970).
13. S. Sander, The *in vitro* uptake of oestradiol in biopsies from twenty-five breast cancer patients. *Acta Path. Microbiol. Scand.* **74,** 301 (1968).
14. P. Feherty, G. Farrer-Brown and A. E. Kellie, Oestradiol receptors in carcinoma and benign disease of the breast: an *in vitro* assay. *Brit. J. Cancer* **25,** 697 (1971).
15. R. Hähnel, E. Twaddle and A. B. Vivian, Oestrogen receptors in human breast cancer. *In vitro* binding of oestradiol by benign and malignant tumours. *Steroids* **18,** 681 (1971).
16. S. G. Korenman and B. A. Dukes, Specific oestrogen binding by the cytoplasm of human breast carcinoma. *J. clin. Endocr.* **30,** 639 (1970).
17. G. Mikhail, C. H. Wu, M. Ferin and R. L. Vande Wiele, Radio-immunoassay of plasma oestrone and oestradiol. *Steroids* **15,** 333 (1970).
18. 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).
19. H. Braunsberg, V. H. T. James, W. T. Irvine and A. E. Carter, Relative oestrogen concentrations in human tumours. *Hormones* **1,** 73 (1970).
20. W. J. Youden, *Statistical Methods for Chemists*, p. 16. John Wiley and Sons, New York (1951).
21. P. Armitage, *Statistical Methods in Medical Research*, p. 97. Blackwell Scientific Publications, Oxford (1971).
22. B. S. Leung, W. Jack and D. C. Wood, Steroid sulfurylation in normal tissues and mammary tumours: a micro method. *Physiol. Chem. & Physics* **4,** 543 (1972).

23. H. Maass, B. Engel, H. Hohmeister, F. Lehmann and G. Trams, Oestrogen receptors in human breast cancer tissue. *Am. J. Obst. Gynecol.* **113,** 377 (1972).
24. B. S. Leung, W. S. Fletcher, T. D. Lindell, D. C. Wood and W. W. Krippaehne, Predictability of response to endocrine ablation in advanced breast carcinoma. *Arch. Surg.* **106,** 515 (1973).
25. J. B. Adams, Formation of steroid sulphates by extracts of human adrenals. *Biochim. Biophys. Acta* **71,** 243 (1963).
26. J. B. Adams, Enzymic synthesis of steroid sulphates I. Sulphation of steroids by human adrenal extracts. *Biochim. biophys. Acta* **82,** 572 (1964).
27. H. Boström and B. Wengle, Studies on ester sulphates. 23. Distribution of phenol and steroid sulphokinase in adult human tissues. *Acta Endocr.* **56,** 691 (1967).
28. R. Dixon, V. Vincent and N. Kase, Biosynthesis of steroid sulphates by normal human testis. *Steroids* **6,** 757 (1965).
29. T. Laatikainen, E. A. Laitinen and R. Vihko, Secretion of free and sulphate-conjugated neutral steroids by the human testis. Effect of administration of human chorionic gonadotrophin. *J. clin. Endocrin.* **32,** 59 (1971).
30. M. A. Rivarola, J. M. Saez, H. W. Jones, G. S. Jones and C. J. Migeon, The secretion of androgens by the normal, polycystic and neoplastic ovaries. *Johns Hopk. med. J.* **121,** 82 (1967).
31. K. Kalliala, T. Laatikainen, T. Luukkainen and R. Vihko, Neutral steroid sulphates in human ovarian vein blood. *J. clin. Endocr.* **30,** 533 (1970).
32. E. Wallace and N. Silberman, Biosynthesis of steroid sulphates by human ovarian tissue. *J. biol. Chem.* **239,** 2809 (1964).
33. E. Melville and H. Braunsberg, Steroid sulphation by human female reproductive tissues *in vitro*. *Biochem. Soc. Trans.* **1,** 757 (1973).
34. E. E. Baulieu, Studies of conjugated 17-ketosteroids in a case of adrenal tumour. *J. clin. Endocr.* **22,** 501 (1962).
35. G. L. Cohn, P. J. Mulrow and V. C. Dunne, *In vitro* synthesis of dehydroepiandrosterone sulphate by an adrenal adenoma. *J. clin. Endocr.* **23,** 671 (1963).
36. M.-C. Lebeau and E. E. Baulieu, *In vitro* biosynthesis of corticosteroid sulphates by adrenal tumour tissue. *Endocrinol.* **73,** 832 (1963).
37. E. Z. Wallace and S. Lieberman, Biosynthesis of dehydroepiandrosterone sulphate by human adrenocortical tissue. *J. clin. Endocr.* **23,** 90 (1963).
38. H. Braunsberg, V. H. T. James, W. T. Irvine, F. James, C. W. Jamieson, R. A. Sellwood, A. E. Carter and M. Hulbert, Prognostic significance of oestrogen uptake by human breast-cancer tissue. *Lancet* **i,** 163 (1973).

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Changes in the Composition of Rat Liver Chromatin Fractions During Nitrosamine Carcinogenesis

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Abstract—*Chromatin fractions have been prepared from the nuclei isolated from the livers of control and diethylnitrosamine (DEN) treated rats (70 days after 3 mg/kg per day). Three fractions containing DNA were obtained after treatment of the nuclei with high pressure followed by ultrasonics.*

The fractions were labelled by injecting the animals with radioactive RNA and DNA precursors. In the carcinogen treated animals given tritiated thymidine a three fold increase in specific activity of the DNA was observed in all fractions.

The non-histone proteins were examined by SDS polyacrylamide gel electrophoresis. A polypeptide with a molecular weight of 17,500 was found to be missing in the euchromatin of DEN treated liver.

INTRODUCTION

IT IS ALMOST certain that some change in the genetic expression of the cell, either by a deletion of a gene or genes and/or a change in chromatin transcription, must occur during transformation to a neoplastic state. In an attempt to elucidate these changes we have been interested in events occurring in the nuclear proteins during carcinogenesis in the rat liver [1]. For these studies the potent carcinogen diethylnitrosamine (DEN) was employed at very low dose levels to minimise the superimposed toxic effects which usually follow the administration of a carcinogen. Druckrey *et al.* [2] have shown that rats given DEN in the drinking water at 3 mg/kg per day develop multifocal liver tumours after about 180 days. The work of Harbers *et al.* [3] has indicated that in this system an increase in the heterochromatin content of the liver occurs after about 40 days of carcinogen feeding; the DNA content per nucleus remained unchanged. These authors used a variation of the method originally described by Frenster, Allfrey and Mirsky [4] to prepare chromatin fractions from interphase calf thymus lymphocytes.

The application of this method to rat liver nuclei, however, was not wholly satisfactory, the sonication step being somewhat critical. In attempting to repeat this work we have experienced similar problems and, as a result have developed a modified procedure in which 90% of the nuclei are ruptured using a very short sonication time. The chromatin fractions subsequently isolated were examined for biochemical differences.

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MATERIAL AND METHODS

Animals

Tuck Wistar SPF rats (supplied by A. Tuck and Son, Rayleigh, Essex) were used throughout. Animals of 180–200 g were divided randomly into two equal groups. The treated were given the DEN in their drinking water (25–30 ppm) at the rate of 3 mg/kg per day.

Groups of 5–6 animals were killed after approximately 70 days; the animals now weighed about 400 g.

Preparation of liver nuclei

All procedures were carried out at 0–4°. Nuclei were prepared by a modification of the Chauveau procedure using 3mM Ca^{2+} as previously described [1]. They were then resuspended in 0·25 M sucrose 3mM Ca^{2+} and a dilution taken for a nuclear count in a modified Neubauer chamber. There was no difference in the yield of nuclei obtained from equal weights of treated and control livers.

Initially nuclei were spun down and resuspended in 0·25 M sucrose (according to the procedure of Frenster *et al.* [4]) at a concentration of 10^7–10^8 nuclei/ml. In some cases the nuclei were centrifuged down again and then resuspended in 0·25 M sucrose as before. After standing for 15–20 min at 4° considerable swelling of the nuclei could be observed under the phase microscope. However, it was found difficult to rupture a large percentage of these nuclei even after long sonication times.

In later experiments it was found that nuclei could be ruptured with greater ease if resuspended in 0·25 M sucrose 10 mM TES buffer (N-tris[Hydroxymethyl]methyl-2-aminoethane sulphonic acid—a non-chelating buffer; supplied by Sigma) at a pH of 7·4 as described by Leake *et al.* [5].

Treatment of nuclei

Initially attempts were made to completely rupture the nuclei by sonication. A Kerry Ultrasonic generator, type KG100 (Chester Hall Lane, Basildon, Essex) fitted with a 6 cm long, 0·9 cm dia probe was used at 100 W (18–22 kHz at 240 V). 10–20 ml volumes of nuclear suspension were sonicated in cooled vessel (0–4°).

In later experiments a cooled Aminco stainless steel French pressure cell (American Instrument Co. Inc. 8030 Georgia Ave., Silver Spring Md.) was used in conjunction with a Wabash Hydraulic Press. 20–40 ml of suspension were subjected to pressures of 8000 p.s.i. (see Busch and Smetana [6]).

Separation of chromatin fractions

Basically the method of Frenster *et al.* [4] was used but the procedure finally adopted is summarized below:

Rat liver nuclei in 0·25 M sucrose 10 mM TES buffer pH 7·4 (approx. 1 mg DNA/ml)

8000 p.s.i. Pressure + 10 sec sonication

500 *g* for 10 min Sediment = Unbroken nuclei

3000 *g* for 30 min = Fraction I ["Heterochromatin"]

75,000 *g* for 60 min = Fraction II ["Intermediate chromatin"]

Precipitated by

10 mM Ca^{2+} = Fraction III ["Light euchromatin"]

Supernatant saturated with ammonium sulphate = Fraction IV ["Soluble nuclear sap proteins"]

Although nucleoli are usually present in the 500 *g* spin when the nuclei are sonicated in 0·25 M sucrose [see 6] they could not be detected in quantity when TES buffer was used.

Injection of labelled precursors

All radio-isotope injections were given at 09.00 hours to avoid complications arising from diurnal variation.

(a) To check the transcriptional activity of the chromatin fractions, animals were injected with the liver RNA precursor tritiated orotic acid (Amersham TRA 83 specific activity 1 Ci/mM). 25 μCi/100 g body weight was injected intraperitoneally in 1 ml of isotonic saline. This was usually given 15 min before killing but similar results were obtained after 30 min.

(b) To monitor DNA synthetic activity of the fractions, animals were given 50 μCi/100 g body weight of tritiated thymidine (Amersham TRA 61, specific activity 5 Ci/mM) intraperitoneally. Animals were killed 60 min later.

Extraction of non-histone proteins

This was achieved by treating the insoluble chromatin fractions with 8 M urea 50 mM phosphate pH 7·6 [7]. Using this procedure the bulk of the non-histone protein is removed leaving the DNA and histone insoluble. Proteins present in the 8 M urea extracts (and in the fractions precipitated by ammonium sulphate) were analysed by SDS electrophoresis in polyacrylamide gel using the technique of Weber and Osborn [8]. Densitometric measurements on Coomassie blue stained gels were made in a modified Joyce–Loebl chromoscan.

DNA was determined by the method of Burton [9] using calf thymus DNA (Sigma) as a standard. Protein was estimated by the Folin Lowry or Buiret methods [10] using bovine serum albumin (Sigma) as a standard. RNA was measured by the Orcinol method [11].

RESULTS

Table 1 shows the effects of various treatments on the composition of the chromatin fractions. All the nuclear DNA was recovered in these fractions. It can be seen that a combination of pressure and a short sonication time disrupted approximately 93% of the nuclei present. It was not possible to achieve this with sonication alone unless undesirably long sonication times were employed.

Table 1. *DNA content of fractions (percentage of total)*

Fraction	Sonication 10 sec	Pressure 8000 p.s.i.	Pressure + sonication 8000 p.s.i. + 10 sec
Unbroken nuclei	38·8	38·7	7·2
3000 *g* (I)	25·0	4·5	10·3
75,000 *g* (II)	33·0	40·6	58·6
Ca^{2+} ppt(III)	3·1	16·0	23·8

10–12 mg of DNA, nuclei suspended in 10 ml of 0·25 M sucrose 10 mM TES pH 7·4.

Table 2. *Percentage DNA contents of chromatin fractions from rat liver nucleic treated by pressure cell* (8000 *p.s.i.*) *and sonication*

	Fraction I	Fraction II	Fraction III
Control	11·8 ± 1·8	64·2 ± 3·5	23·8 ± 1·9
DEN treated	8·6 ± 5·4	69·7 ± 6·0	21·6 ± 5·1

I ("Heterochromatin") = 3000 *g* for 30 min.
II ("Intermediate Chromatin") = 75,000 *g* for 60 min.
III ("Light Euchromatin") = Ca^{2+} precipitate.
Mean of 6 groups each containing 5–6 animals ± S.D.

Differences in the composition of chromatin fractions prepared from control and DEN treated animals (approximately 70 days) are shown in the Table 2. Contrary to the findings of Harbers *et al.* [3] we did not, using our technique, find an increase in the so-called "heterochromatin" fraction (fraction I). In fact a slight, but variable, decrease in this fraction was observed (not significant, according to a Student *t*-test, $P = 0{\cdot}2$) but there was no outstanding differences in composition between control and treated samples. When this technique was applied to nuclei isolated from hepatoma 223 ascites cells (a butter-yellow derived tumour) fraction I contained 8·5%, II 61·5% and III 30·0% of the DNA. Rat thymus nuclei gave values of 47·0, 47·5 and 5·5 respectively.

From Table 3 it is evident that fraction III (i.e. that precipitated by 10 mM Ca^{2+}) contains the most newly labelled RNA and is therefore the nearest to the definition of euchromatin. The specific activity of the RNA in fraction III was slightly higher than I and II but there was no difference between control and treated fractions or in the specific activity of whole nuclear RNA.

Table 3. *Specific activity of chromatin fractions (prepared by pressure and sonication) 15 min pulse of* ^3H*–orotic acid* (25 *μCi*/100*g body wt*)

	S.A. of Chromatin fractions dpm/*μg* DNA)			
	Whole nuclei	I	II	III
Control	176	133	86	250
DEN treated (70 days)	192	115	71	271

Fractions as in Table 2.

Table 4. *Specific activity of chromatin fractions (prepared by pressure and sonication) 60 min after administration of* ^3H*–thymidine*

	Specific activity of chromatin fraction (dpm/mg DNA)		
	I	II	III
Control	8250	7369	6475
DEN treated (70 days)	30300	26649	20696

Fractions as in Table 2.

The results obtained after labelling the DNA of the fractions with tritiated thymidine are shown in Table 4. Although there is a slight difference in the specific activity of the control fractions a very notable increase occurs in the fractions obtained from the DEN treated animals. This increased incorporation of tritiated thymidine into the liver DNA of DEN treated rats (given 5 mg/kg per day in their drinking water) has been detected by autoradiography [12]. It has also been observed in the liver of rats 4 weeks after a single carcinogenic dose of dimethylnitrosamine (DMN) [1].

The SDS polyacrylamide gel patterns

obtained from the non-histone proteins of the chromatin fractions are shown in Fig. 1. The patterns from fractions I, II and III appear to contain more lower molecular weight polypeptide material than is evident when whole nuclear 8 M urea extracts are examined (e.g. see [7]). This is due to the fact that the main bulk (approximately 50–60%) of the nuclear 8 M urea soluble protein material is present in fraction IV which contains an abundance of the higher molecular weight components. It can be seen that there is very little difference between (C) and (T) samples as judged by the Coomassie Blue dye binding patterns. The exception is fraction III where a band (marked by an arrow in Fig. 1) is seen to be either reduced in quantity or missing in the treated samples. Gels were run from 3 separate chromatin protein preparations and showed the band in the control sample to have a mobility of $0{\cdot}65 \pm 0{\cdot}01$ [8]. A molecular weight curve was constructed from the mobilities of 15 polypeptides of known molecular weight. Using this, a value of 17,500 was obtained for the protein missing in the DEN treated fraction III.

We have examined densitometric traces of the gels shown in Fig. 1, and in fraction II (gels 3 and 4) there is no quantitative difference in dye intensity of the band of mobility 0·65 in control and treated samples. This band was not present in fraction I or IV samples.

To check for the presence of this missing component in the 8 M urea insoluble residues of the treated chromatin, the following procedure was carried out:

The isolated chromatin fractions were dissolved in 8 M urea, 1% SDS, 50 mM tris-HCl pH 8, containing 0·1% β-mercaptoethanol. The DNA was sedimented by centrifugation at 150,000 *g* for 16 hr, the top two-thirds of solution (containing protein) was dialysed against starting buffer and subjected to SDS gel electrophoresis. Since this procedure solubilises the histones, whereas the 8 M urea extraction procedure does not (details unpublished) the gels showed a preponderance of low molecular weight components. However, in the case of the treated liver fraction III, there was a considerable decrease in the content of bound dye in the region corresponding to mobility of 0·65. This was not evident in preparations from fraction II.

Similarly, if the urea extracted chromatin preparations were subjected to this procedure, it could be shown that there was no difference between treated and control samples in the dye bound in the region of the missing band.

DISCUSSION

Although from the evidence of molecular cytology etc. the existence of hetero- and euchromatin in the interphase differentiated cell is a well documented phenomenon, satisfactory biochemical methods to separate out these chromatin fractions have not been developed. The most commonly employed method is the use of ultrasonics on nuclei preswollen in cation-free medium followed by sedimentation of the fractions by differential centrifugation. This method was originally described by Frenster, Allfrey and Mirsky [4] and was applied to nuclei isolated from interphase calf thymus lymphocytes. Attempts have been made by various other workers to apply this method to isolated rat liver nuclei [3, 13, 20] but a clear separation of eu- from heterochromatin is difficult to achieve [e.g. 14]. Although in the experiments we have described here fractions I and II contained considerable amounts of newly synthesised RNA, fraction III had about 2–3 times more RNA associated with the DNA. Despite the fact that the specific activity was not a great deal higher than in the other two fractions, fraction III is probably the richer in euchromatic fragments.

The 3–4-fold increase in ^{3}H-Tdr uptake in the liver DNA of the carcinogen treated animals is interesting. Undoubtedly some of this might be involved in the repair of alkylated DNA which is produced by nitrosamine treatment [15]. Changes in the pool size of the treated animals may also contribute to this rise in ^{3}H-Tdr uptake but this is unlikely to be a major factor since several workers have reported that an increased level of polyploidization occurs in the liver nuclei of DEN treated rats [16, 17]. However, Rajewsky [12] has pointed out that this does not appear high enough to explain the increased ^{3}H-Tdr labelling index.

The significance of these findings in relation to carcinogenic change remains to be elucidated but the concomitant loss or large decrease in a non-histone protein with a molecular weight of 17,500 from fraction III could be an important factor in the increased DNA synthesis. Apart from this effect little change in the composition of the non-histone or "acidic" nuclear protein fraction seems to be noticeable. In fact it is difficult to observe the loss of this polypeptide if the 8 M urea extracts of nuclei alone are examined by SDS electrophoresis but it can just be seen in the densitometric traces of some gel samples.

The role of nuclear proteins in the control of

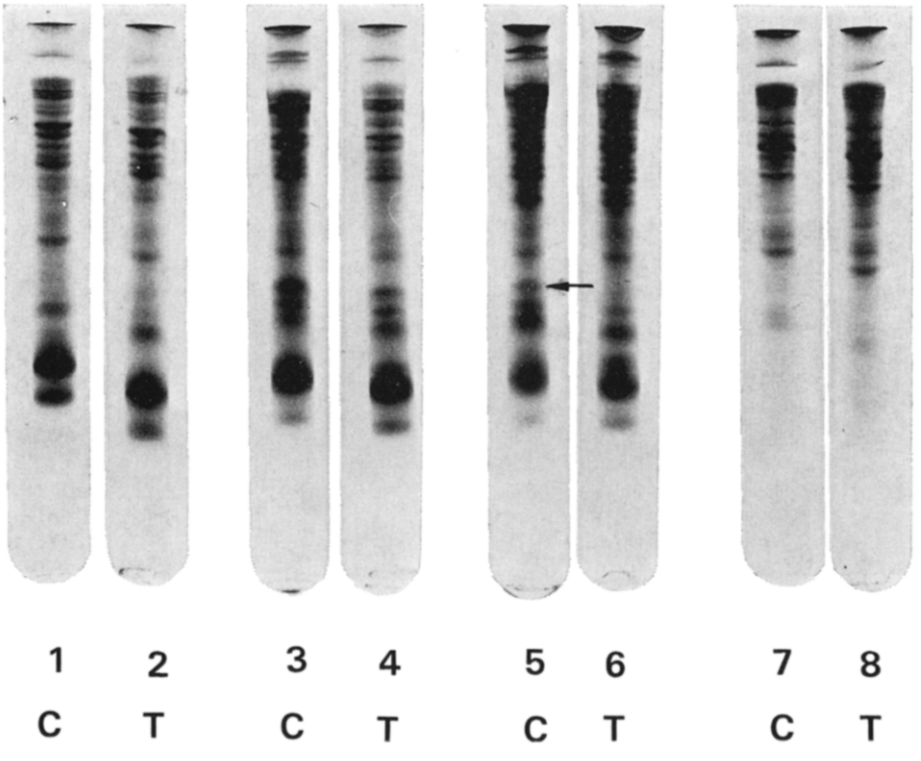


Fig. 1. *SDS polyacrylamide gel electrophoresis of the non-histone proteins of the chromatin fractions (stained with Coomassie Brilliant Blue).*

C = from control livers
T = from DEN treated livers
1 & 2 = *Heterochromatin (I)*
3 & 4 = *Intermediate chromatin (II)*
5 & 6 = *Light Euchromatin (III)*
7 & 8 = *Soluble nuclear sap proteins (IV)*

(To face p. 24)

transcription and neoplastic change is still unknown (see [18] for review). Recent improvements in analytical techniques [19] now make these investigations possible. Elucidation of the biological role of the missing polypeptide observed in our experiments must therefore await its isolation from the chromatin of normal tissues in sufficient quantities for biochemical analysis.

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REFERENCES

1. M. Gronow, Imbalances in DNA and histone synthesis in the rat liver during neonatal carcinogenesis induced by dimethylnitrosamine. *Europ. J. Cancer* **7,** 341 (1971).
2. H. Druckrey, A. Schildbach, D. Schmähl, R. Preussmann and S. Ivankovic. Quantitative Analyse der carcinogenen Wirkung von Diäthylnitrosamin. *Arzneimittel-Forsch* **13,** 841 (1963).
3. E. Harbers, B. Lederer, W. Sandritter and U. Spaar, Untersuchungen an Nucleohistonen. IV. Heterochromatisierung in der Rattenleber während der Carcinogenese. *Virchows. Arch. Abt. B. Zellpath* **1,** 98 (1968).
4. J. H. Frenster, V. G. Allfrey, and A. E. Mirsky, Repressed and active chromatin isolated from interphase lymphocytes. *Proc. nat. Acad. Sci. (Wash.)* **50,** 1026 (1963).
5. R. E. Leake, M. E. Trench and J. M. Barry, Effect of cations on the condensation of hen erythrocyte nuclei and its relation to gene activation. *Exp. Cell Res.* **71,** 17 (1972).
6. A. Busch and K. Smetana, In *The Nucleolus.* Academic Press, New York (1970).
7. M. Gronow and G. Griffiths, Rapid isolation and separation of the non-histone proteins of rat liver nuclei. *FEBS Lett.* **15,** 340 (1971).
8. K. Weber and M. Osborn, The reliability of molecular weight determination by dodecyl sulphate—polyacrylamide gel electrophoresis. *J. biol. Chem.* **244,** 4406 (1969).
9. K. Burton, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. *Biochem. J.* **62,** 315 (1956).
10. E. Layne, In *Methods in Enzymology III.* (Edited by S. P. Colowick and N. O. Kaplan) pp. 447 (1957). Academic Press, New York.
11. G. Ceriotti, Determination of nucleic acids in animal tissues. *J. biol. Chem.* **214,** 59 (1955).
12. M. F. Rajewsky, Changes in DNA synthesis and cell proliferation during hepatocarcinogenesis by diethylnitrosamine. *Europ. J. Cancer* **3,** 335 (1967).
13. F. Dolbeare and A. Koenig, Fractionation of rat liver chromatins: effects of cations, hepatectomy and actinomycin D. *Proc. Soc. exp. Biol (N.Y.)* **135,** 636. (1970).
14. R. H. De Bellis, W. Benjamin and A. Gelhorn, Studies on *in vitro* RNA synthesis by chromatin fractions from rat liver and Novikoff hepatoma. *Biochem. biophys. Res. Comms.* **36,** 166 (1969).
15. P. N. Magee, *In vivo* reactions of nitroso compounds. *Ann. N.Y. Acad. Sci.* **163** (art. 2) 717 (1969).
16. H. P. Hobik and E. Grundmann, Quantitative Veranderungen der DNS und der RNS in der Rattenleberzelle während der Carcinogenese durch Diäthylnitrosamin. *Beitr. path. Anat.* **127,** 25 (1962).
17. L. Wiest, The effect of diethylnitrosamine on the distribution of cell classes in the parenchyma of the liver of newborn rats. *Europ. J. Cancer* **8,** 121–125 (1972).
18. A. J. MacGillivray, J. Paul and G. Threfall, Transcriptional regulation in eukaryotic cells. *Adv. in Cancer Res.* **15,** 93 (1972).
19. L. S. Hnilica, In *The Structure and Biological Function of Histones.* Chemical Rubber Co., Cleveland, Ohio (1972).
20. R. K. Salganik, T. M. Morozova and M. A. Zakharov, The changes in the state of rat liver chromatin under hydrocortisone induction. *Biochim. biophys. Acta* **174,** 755 (1969).

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Enhancement of the Cancer Chemotherapeutic Effect of the Cell Cycle Phase Specific Agents Methotrexate and Cytosine Arabinoside when given as a Water–Oil–Water Emulsion

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Abstract—*Parenteral administration of methotrexate as a water–oil–water emulsion enhances its therapeutic activity against the mouse R1 lymphoma and rat Walker tumour.*

Methotrexate incorporated into a water–oil–water emulsion based on Squalane as the oil exhibited the greatest therapeutic activity. A single dose of 3 mg/kg in the emulsion form gave a greater prolongation of survival time of mice bearing the R1 lymphoma than five daily doses (total dose, 15 mg/kg) of methotrexate in aqueous solution.

A chemotherapeutic effect similar to that of five daily doses of cytosine arabinoside in aqueous solution could also be obtained against the R1 lymphoma by a single dose of this drug in a water–oil–water emulsion formulation.

INTRODUCTION

ELSON *et al.*, 1970 [1], described how the administration of methotrexate in a water–oil–water emulsion based on light liquid paraffin as the oil, enhanced its therapeutic activity against the L1210 leukaemia in mice. A single dose of 3 mg/kg methotrexate in the emulsion form gave a much longer survival of the leukaemic mice than a single dose of 80 mg/kg of aqueous methotrexate, and a slightly greater therapeutic effect than five daily doses of 3 mg/kg (total dose 15 mg/kg) of methotrexate, also in aqueous solution. The sodium salt of methotrexate was used in all cases. It was also shown that altering the composition of the water–oil–water emulsion modified the therapeutic activity achieved.

Since these preliminary results appeared so promising, further development of this method of administration leading to its possible clinical application was carried out. Because of the possible objections to the clinical use of light liquid paraffin by injection, its replacement by another emulsifying base, squalane, was investigated.

A report is now presented on the enhancement of the cancer chemotherapeutic activity of methotrexate sodium against the R1 lymphoma in mice, and the Walker 256 tumour in rats, and of cytosine arabinoside against the R1 lymphoma in mice, by their inclusion in water–oil–water emulsion formulations.

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MATERIAL AND METHODS

Preparation of the water–oil–water emulsion

The exact procedure for the preparation of the water–oil–water emulsion has already been described [1]. Firstly, a water in oil emulsion was prepared, the aqueous phase of this emulsion containing either methotrexate sodium or cytosine arabinoside. An aliquot of this emulsion was then added to an equal volume of a 2% v/v solution of Tween 80 in distilled water, and shaken gently by hand for 30 sec to form the water–oil–water emulsion.

In some of the emulsions described here, light liquid paraffin was substituted by squalane (2,6, 10,15,19,25-hexamethyltetracosane, BDH), and the emulsifying agent Arlacel 83 (sorbitan sesquioleate) by the more refined Arlacel A (mannide monooleate).

The composition of the water–oil–water emulsion formulations was altered by varying the three basic parameters of the water in oil emulsion: the osmolarity of the internal aqueous phase, (varied by the inclusion of different quantities of sodium chloride), the concentration of emulsifying agent, and the internal phase volume.

Assay of the inhibitory action on the growth of the R1 lymphoma in mice

Female CBA/LAC mice were injected subcutaneously with a suspension of approximately 1×10^6 ascites cells of the R1 lymphoma. Methotrexate or cytosine arabinoside treatment by intraperitoneal injection was commenced 72 hr after implantation of the tumour. The volume of drug in aqueous solution or in water–oil–water emulsion formulation injected was 0·2 ml/20 g mouse. The mice were observed daily and the day of death for individual mice was recorded. Control experiments were carried out in which the mice did not receive methotrexate or cytosine arabinoside, but received an injection of saline. Mice were also injected with a water–oil–water emulsion which did not contain any drug.

Table 1. *The effect of I. a single aqueous dose, II. five daily aqueous doses of methotrexate, and III. a water–oil–water emulsion formulation based on light liquid paraffin containing methotrexate on the survival of mice bearing R1 lymphoma*

		(Average of 10 mice)		
	Total dose (mg/kg)	Average day of death of untreated control	Average day of death	Increase in survival time over untreated control (%)
I.	3·75	10·0	12·2	22·0
	7·5	10·0	11·8	18·0
	15·0	10·0	12·8	28·0
	30·0	10·0	12·0	20·0
	60·0	10·0	12·6	26·0
	120·0	10·0	12·8	28·0
	240·0	10·2	12·6	23·5
II.	0·9375	9·5	10·6	11·6
	1·875	9·5	11·0	15·8
	3·75	9·5	12·0	26·3
	7·5	9·8	16·0	63·3
	15·0	9·8	17·0	73·5
	30·0	9·8	16·2	65·3
	60·0	9·8	12·2	24·5
	120·0	10·2	9·0	−11·8
III.	0·1875	10·2	10·0	−2·0
	0·375	10·2	10·2	0
	0·75	10·2	10·6	3·9
	1·5	10·2	13·6	33·3
	3·0	10·2	16·4	60·8
	6·0	10·2	17·0	66·7
	12·0	10·2	11·8	15·7
	24·0	10·2	8·4	−17·6

Water in oil emulsion: 10% Arlacel 83, 2% sodium chloride, 10% internal phase volume.

Assay of the inhibitory action on the growth of the Walker 256 *tumour in rats*

The procedure for the assessment of the inhibitory action of methotrexate on the Walker 256 tumour in rats has been described earlier [7]. Drug treatment by intraperitoneal injection was commenced 24 hr after implantation of the tumour. The volume of drug in aqueous solution or in a water–oil–water emulsion formulation injected was 1 ml/100 g rat. Control experiments were carried out in which the rats did not receive methotrexate, but received an injection of saline. The rats were also injected with a water–oil–water emulsion which did not contain any methotrexate.

RESULTS

1. *Methotrexate sodium*

Table 1 shows the effect of methotrexate on the survival of mice bearing the R1 lymphoma. When given as a single aqueous dose of 3·75 mg/kg, methotrexate increased the survival time of the animals by 22%, and with progressively increased doses (up to 240 mg/kg) there was no further improvement in its therapeutic efficiency. However, when 3 mg/kg methotrexate was given daily for five consecutive days (total dose, 1·5 mg/kg), a 74% prolongation in survival times was achieved.

Table 1 also shows the effect of a methotrexate containing water–oil–water emulsion formulation based on light liquid paraffin on the survival of the mice. A single dose of 3 mg/kg in this emulsion formulation increased the survival time of the animals by 61%. When the water–oil–water emulsion vehicle was administered, no increase in survival time of the mice was noted.

The dose response relationships of these different forms of administration are shown in Fig. 1. It can be seen that the single dose of 3 mg/kg or 6 mg/kg of methotrexate in the emulsion formulation gave a very similar result to five daily doses of 3 mg/kg (total dose 15 mg/kg) or 6 mg/kg (total dose 30 mg/kg) in aqueous solution.

The efficiency of single doses of 3 mg/kg of methotrexate on prolonging the survival of mice bearing the R1 lymphoma when given as twelve different water–oil–water emulsion formulations based on squalane as the oil was investigated. The composition of the formulations that were tested and the results obtained are compared in Table 2. It has been shown that modification of the formulation of the water in oil emulsion can modify the effectiveness of the water–oil–water emulsion formulation [6]. The parameters which have been investigated here are the osmolarity of the internal aqueous phase, (EMS 23–25), the concentration of emulsifying agent used (EMS 23, 26 and 27), and the internal phase volume (EMS 23, and 28–34). The most striking factor which affected chemotherapeutic activity was the internal phase volume of the primary water in oil emulsion. The most efficient formulation was EMS 32 where the internal phase volume was 30%. With this formulation a 106% increase in survival time was obtained, which was an improvement on the 74% increase achieved with five daily doses of an aqueous solution of the drug. Raising the internal phase volume to 60%, or lowering it to 10% reduced the chemotherapeutic activity of the formulation.

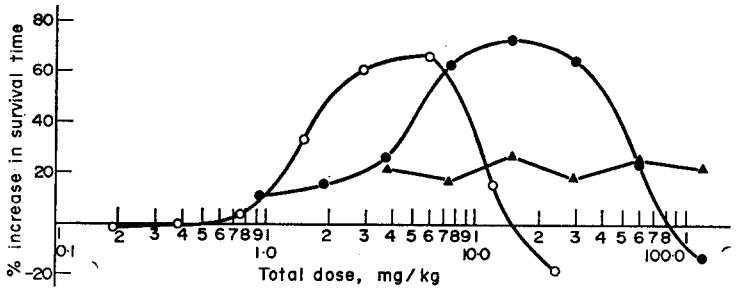


Fig. 1 The effect of methotrexate in aqueous solution and in an emulsion formulation based on light liquid paraffin on the survival of mice bearing the R1 lymphoma.

This optimum emulsion formulation, EMS 32, was tested for its effect in inhibiting the growth of the Walker 256 tumour in rats, and again compared with the effect of five daily doses of aqueous methotrexate (Table 3). It can be seen that these two forms of medication were of similar biological activity, although the total emulsion dose of methotrexate was again only about one fifth of the total aqueous dose. Administration of this water–oil–water emulsion vehicle did not cause any inhibition in the growth of the tumour.

Table 2. The effect of a single dose of 3 mg/kg of methotrexate in water–oil–water emulsions based on squalane on the survival of mice bearing the R1 lymphoma

Emulsion Code No.	Sodium chloride (%)	Primary detergent (Arlacel A) (%)	Internal phase volume (%)	Average survival time (days) (Average of 10 mice)	Increase in survival time over untreated control (%)
Control				10·0	
EMS 24	2	10	50	11·0	10·0
EMS 25	5	10	50	11·2	12·0
EMS 26	2	5	50	11·4	14·0
EMS 27	2	15	50	11·2	12·0
EMS 28	2	10	25	11·8	18·0
EMS 29	2	10	10	13·2	32·0
Control				9·8	
EMS 23	0	10	50	18·2	85·7
EMS 30	0	10	60	16·6	69·4
EMS 31	0	10	40	19·4	98·0
EMS 32	0	10	30	20·2	106·1
EMS 33	0	10	20	17·4	77·6
EMS 34	0	10	10	12·4	26·5

Table 3. The effect of I. five daily doses of methotrexate in aqueous solution and II. a water–oil–water emulsion formulation based on squalane containing methotrexate on the Walker 256 tumour in rats

	Methotrexate Dose mg/kg	No. of doses	Total dose (mg/kg)	Average tumour weight (g) (Average of 6 rats)	Average tumour weight (% control)	Inhibition of tumour (%)
I.	Aqueous					
	Control			42·8		
	0·075	5	0·375	39·0	91·1	8·9
	0·15	5	0·75	40·3	94·2	5·8
	0·3	5	1·5	32·3	75·5	24·5
	0·6	5	3·0	12·3	28·8	71·2
	1·2	5	6·0	5·0	11·6	88·4
	2·4	5	12·0	1·6	3·9	96·1
II.	Emulsion					
	Control			42·8		
	0·0625	1	0·0625	40·6	94·8	5·2
	0·125	1	0·125	37·0	86·4	13·6
	0·25	1	0·25	32·3	75·4	24·6
	0·5	1	0·5	27·3	63·7	36·3
	1·0	1	1·0	10·0	23·3	76·7
	2·0	1	2·0	0·0	0·0	100·0

Water in oil emulsion: 10% Arlacel A.
No sodium chloride.
30% internal phase volume.

II. *Cytosine arabinoside*

Table 4 shows the effect of cytosine arabinoside in aqueous solution on the survival of mice bearing R1 lymphoma. A very similar pattern to methotrexate treatment was obtained in that increasing single doses of the drug had very little effect on the survival of the animals, but if the dose was repeated daily for five days, a considerable prolongation in the life span of the mice could be achieved. 200 mg/kg cytosine arabinoside given as a single aqueous injection gave only a 4·1% prolongation of survival time of the mice, but when this dose was repeated daily for five days, a 67·0% prolongation was achieved. Administration of a water–oil–water emulsion formulation alone had no effect on the survival time of the mice. When the drug was incorporated into the water–oil–water emulsion formulation, a dose of 200 mg/kg gave a prolongation of 60·8%, again approximately equiactive with five daily doses of the aqueous solution. The detailed results obtained with the water–oil–water emulsion formulation are also shown in Table 4. Figure 2 shows a comparison of the dose–response relationships of the three different means of administering cytosine arabinoside. It shows that a single injection of the drug in a water–oil–water emulsion formulation gives results of similar biological effectiveness to repeated aqueous injections, and that the emulsion dose was only one fifth of the amount of drug administered in aqueous solution.

Table 4. The effect of I. a single aqueous dose, II. five daily aqueous doses of cytosine arabinoside, and III. a water–oil–water emulsion formulation based on squalane containing cytosine arabinoside on the survival of mice bearing the R1 lymphoma

		(Average of 10 mice)		
	Total dose (mg/kg)	Average day of death of untreated control	Average day of death	Increase in survival time over untreated control (%)
I.	12·5	9·8	9·4	−4·1
	25·0	9·8	9·8	
	50·0	9·8	10·4	6·1
	100·0	9·8	10·4	6·1
	200·0	9·8	10·2	4·1
	400·0	9·8	12·6	28·6
II.	65·5	9·7	11·2	15·5
	125·0	9·7	12·6	29·9
	250·0	9·7	13·8	42·3
	500·0	9·7	15·2	56·7
	1000·0	9·7	16·2	67·0
	2000·0	9·7	9·8	1·0
III.	12·5	10·2	11·2	9·8
	25·0	10·2	12·4	21·6
	50·0	10·2	13·8	25·3
	100·0	10·2	14·8	45·1
	200·0	10·2	16·4	60·8

Water in oil emulsion: 10% Arlacel A, 2% sodium chloride, 50% internal phase volume.

DISCUSSION

The water–oil–water emulsion formulation has been shown to give a sustained pharmacological action because of the slow release of the drug in its water soluble form from within the multiple droplets [2]. It has been demonstrated [1] that by prolonging the action of methotrexate by including it in a water–oil–water emulsion formulation, a considerable enhancement of the activity of a single dose of the drug on the survival of mice bearing the L1210 leukaemia could be achieved.

Squalane has been studied here as an alternative oil to light liquid paraffin for the formulation of methotrexate because of objections to the injection of light liquid paraffin in the form of an oily vaccine adjuvant. It was proposed as an alternative oil for this purpose [3]. Squalane is a colourless, odourless, inert liquid, which is fully saturated and therefore not liable to

rancidity, and is a component of human sebum. It has been subjected to stringent toxicity testing and has been found to be non toxic following injection [4]. It appears from the experiments described here that methotrexate emulsion formulations based on squalane as the oil show a greater therapeutic effect than those based on light liquid paraffin.

Arlacel A was substituted for Arlacel 83 as the primary emulsifying agent because it had very little irritant action at the site of injection. It was also used by Herbert [5] who first proposed the water–oil–water emulsion formulation as a vaccine adjuvant instead of a water in oil emulsion because of its greater ease of injection.

In the work presented here, it has been demonstrated that a single dose of methotrexate contained in several different water–oil–water emulsion formulations was as effective, or more effective in prolonging the life of mice implanted with the R1 lymphoma than five daily injections of the drug in aqueous solution at the same dose level, and more effective than a single large dose of the drug in aqueous solution, 80 times that of the emulsion dose. A single dose of methotrexate in an emulsion formulation was also found to be as effective as five daily doses of the drug in aqueous solution at the same dose level in preventing the growth of the Walker 256 tumour in rats.

It was shown that by varying three parameters of the water in oil emulsion, the osmolarity of the internal aqueous phase, the concentration of the primary emulsifying agent, and the internal phase volume, the therapeutic effect of methotrexate in the water–oil–water emulsion formulation could be varied. Collings and Schneider [6] also showed that varying these parameters altered the release rate of the active principle from the formulation.

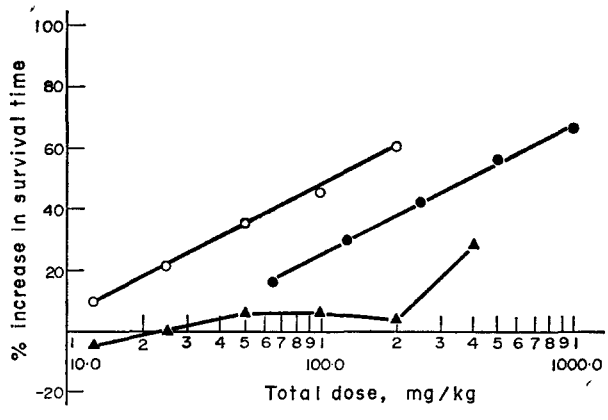


Fig. 2 The effect of cytosine arabinoside in aqueous solution and in an emulsion formulation based on squalane on the survival of mice bearing the R1 lymphoma

This paper also reports the enhanced activity of a water–oil–water emulsion formulation containing cytosine arabinoside based on squalane as the oil over five daily injections of the drug in aqueous solution at the same dose level on the R1 lymphoma in mice. A preliminary test on a water–oil–water emulsion formulation containing vinblastine sulphate against the Erlich Ascites tumour has also been carried out. Further tests with this drug are at present being performed for the results seemed most promising.

REFERENCES

1. L. A. Elson, B. C. V. Mitchley, A. J. Collings and R. Schneider, Chemotherapeutic effect of a water–oil–water emulsion on the mouse L1210 leukaemia. *Rev. Europ. Etud. clin. et biol.* **xv**, 87 (1970).
2. U.K. Patent application numbers 33255/67, 23146/68 and 56421/69.
3. L. B. Holt, Oily adjuvants. International Symposium on Adjuvants of Immunity. Utrecht Symposium Series. Standard Vol. 6, 131 (1966).
4. M. L. Rosenthal, Biological role and practical uses of squalene and squalane. In *Cosmetics and the Skin.* (Edited by I. Lubowe and F. V. Wells.) Reinhold, New York 646 (1964).

5. W. J. HERBERT, Multiple emulsions, a new form of mineral oil antigen adjuvant. *Lancet* **ii,** 771 (1965).
6. A. J. COLLINGS and R. SCHNEIDER, The multiple emulsion formulation for the slow release of drugs. *Brit. J. Pharmac.* **38,** No. 2, 469 (1970).
7. V. M. ROSENOER, B. C. V. MITCHLEY, F. J. C. ROE and T. A. CONNORS, Walker Carcinosarcoma 256 in study of anticancer agents. I. Method for simultaneous assessment of therapeutic value and toxicity. *Cancer Res. Suppl.* **26** (Part 4), 937 (1966).

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Histological and Hormonal Studies in Rats Bearing Ovarian Implants in the Spleen. Effects of Portacaval Anastomosis

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Abstract—*Ovaries were implanted into the spleens of ovariectomized rats. Plasma FSH levels increased rapidly after implantation and this was followed by an increase in plasma concentrations of LH. These raised levels were sustained for at least* 140 *days after operation. Plasma prolactin levels showed no change. Portacaval anastomosis prevented the increases in plasma hormone concentrations, blocked the growth of fresh implants and caused atrophy of established implants.*

INTRODUCTION

BISKIND and Biskind [1] first documented the changes which occur in ovarian implants into the spleen of ovariectomized rats. The implants were found to enlarge considerably and to undergo a sequence of histological changes culminating in the formation of masses of theca cells, "granulosa cell" tumours being found in some of the animals. Similar findings have been reported in rats and rabbits [2–4] in guinea pigs [5] and in mice [6, 7, 8].

The mechanism for such growth is believed one of over production of pituitary gonadotrophins following inactivation of ovarian oestrogens in the liver [1]. The availability of radioimmunoassays for rat pituitary hormones now allows precise measurements to be made of serial changes in plasma gonadotrophins following splenic implantation of ovarian tissue.

It has also been suggested that failure of some implants to undergo hyperplasia and hypertrophy may be related to the development of adhesions between the spleen and parietal peritoneum giving rise to a portal-systemic collateral circulation [2, 8, 9].

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This paper reports an investigation into the histological and hormonal changes following the implantation of ovarian tissue into the spleen of rats. The effect of portacaval anastomosis on the growth of ovarian implants was also examined.

MATERIALS AND METHODS

Ten-week-old, female Sprague–Dawley rats were used. They were fed on rat diet 41B and water *ad libitum.*

Operative technique

Anaesthesia was induced with ether and, in most cases, continued with a single intraperitoneal injection of veterinary Nembutal (0·07 ml/100 g body weight).

Ovariectomy and implantation were carried out through a ventral, mid-abdominal incision. The ovaries were removed after ligation of their vessels, adherent fat and the oviducts discarded and the left ovary halved. The spleen was gently mobilized and an incision made in the capsule of its gastric surface. The capsule was then elevated and one half of the left ovary placed within the "pocket". A flap of omentum was sutured around implant and spleen to minimise the formation of adhesions between the spleen and parietal peritoneum.

End to side portacaval shunting was carried out by a modification of the method of Bismuth *et al.* [10]. The portal vein was divided high at the hilum of the liver and anastomised (end to side) to the superior vena cava, using 7/0 silk mounted on a 12 mm atraumatic needle.

The animals were killed at intervals after operation. A post-mortem examination was carried out and the presence of any adhesions between the spleen and parietal peritoneum noted.

Blood samples

Blood was taken from the external jugular vein (with the rats under ether anaesthesia) or by decapitation of the conscious animal. Samples were placed in tubes containing EDTA. The plasma was then separated by centrifugation and stored at −20°C until assay.

Measurement of plasma hormones

Immunoreactive prolactin, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured in plasma samples by double antibody radioimmunoassays using kits distributed by the National Institute for Arthritis and Metabolic Diseases (NIAMD), Bethesda, Md., U.S.A. The assay protocols were essentially similar to those used previously (11).

Splenic implantation of ovarian tissue with simultaneous bilateral ovariectomy

Pairs of rats from a group (Ia) of 75 animals were killed at intervals for up to 180 days after operation and tissue taken for histological study.

Blood was taken under ether anaesthesia from a group (Ib) of animals before implantation and by decapitation 1 day, 7 days, 9 days, or 20 days after implantation. In another experiment, a group (Ic) of animals were bled under ether anaesthesia before implantation and the same animals bled repeatedly at intervals from 20 to 140 days after implantation.

Splenic implantation of ovarian tissue with delayed bilateral ovariectomy

Ovarian implantation was performed in a group (II) of rats but the right ovary was left *in situ*. Twenty-five days later, the remaining ovary was removed. The animals were killed 25 days after total ovariectomy.

Portacaval shunt followed by splenic implantation of ovarian tissue and bilateral ovariectomy.

A portacaval shunt was performed on a group (III) of 24 animals. Three to five days later, half of the left ovary was implanted into the spleen and the rat ovariectomized. Eighty per cent of the animals survived both operations. The animals were killed in pairs daily from 1 to 14 days and weekly from the 14 to the 77th day after implantation.

Splenic implantation of ovarian tissue with bilateral ovariectomy and a delayed portacaval shunt

Ovarian implants were performed in a group (IV) of 10 rats. A portacaval shunt was then performed 15–25 weeks after implantation at which time measurements were made of the exterior length and width of the implant protruding from the spleen. Eighty per cent of the animals survived both procedures. The animals were killed 4 weeks after portacaval shunting and the size of the implants again measured. In four control rats, laparotomy alone was performed at 15–25 weeks and the size of the implant measured. Animals were killed 4 weeks later and the size of the implants again measured.

RESULTS

Splenic implantation of ovarian tissue with simultaneous bilateral ovariectomy

Histological examination of the implants revealed a characteristic series of changes commencing with a period of necrosis and inflammation in the first days after implantation and progressing to the formation of discrete corpora lutea (Fig. 1). Luteomata or disorganised luteal cells similar to those described by Biskind and Biskind [1] were not observed within the time limits of the experiment (180 days).

Plasma levels of FSH rose rapidly after operation and remained elevated throughout the experimental period. Increases in plasma LH occurred more slowly but again the concentrations were sustained at a high level thereafter. Plasma prolactin levels tended to fall after operation but the changes were not statistically significant (Tables 1 and 2).

Splenic implantation of ovarian tissue with delayed bilateral ovariectomy

Removal of the contralateral ovary 25 days after hemiovariectomy and ovarian implantation produced hyperplasia of the implants after

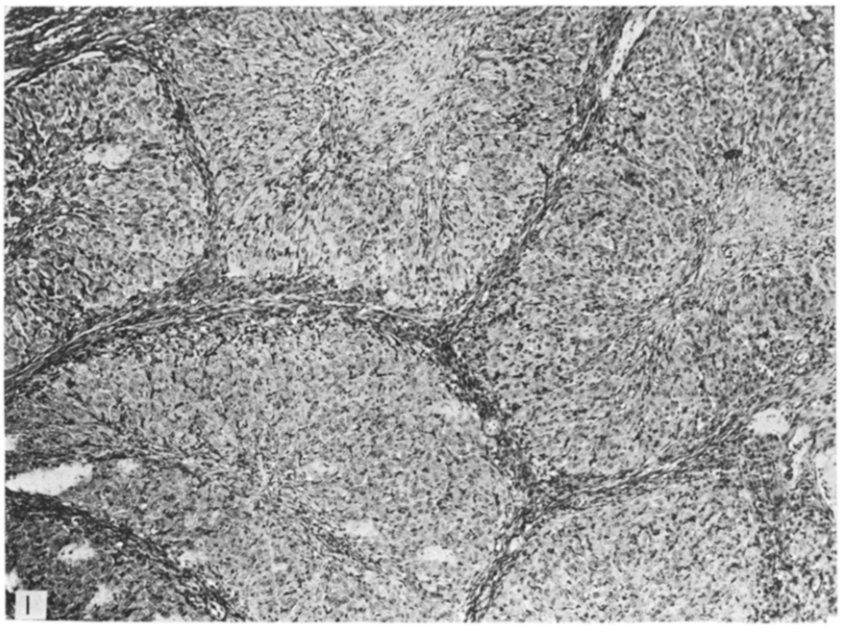


Fig. 1. *Splenic implantation of ovarian tissue and simultaneous bilateral ovariectomy (Group Ia). Massed corpora lutea in an implant* 49 *days after operation. H. & E.* × 100

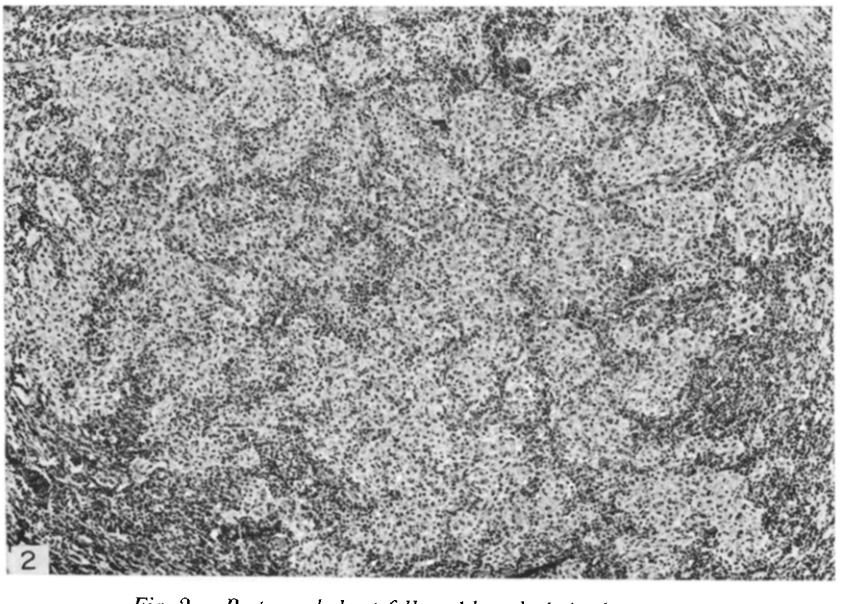


Fig. 2. *Portacaval shunt followed by splenic implantation of ovarian tissue with bilateral ovariectomy (Group III). Diffuse luteal cells in an implant* 14 *days after implantation. H. & E.* × 126.

(to face p. 36)

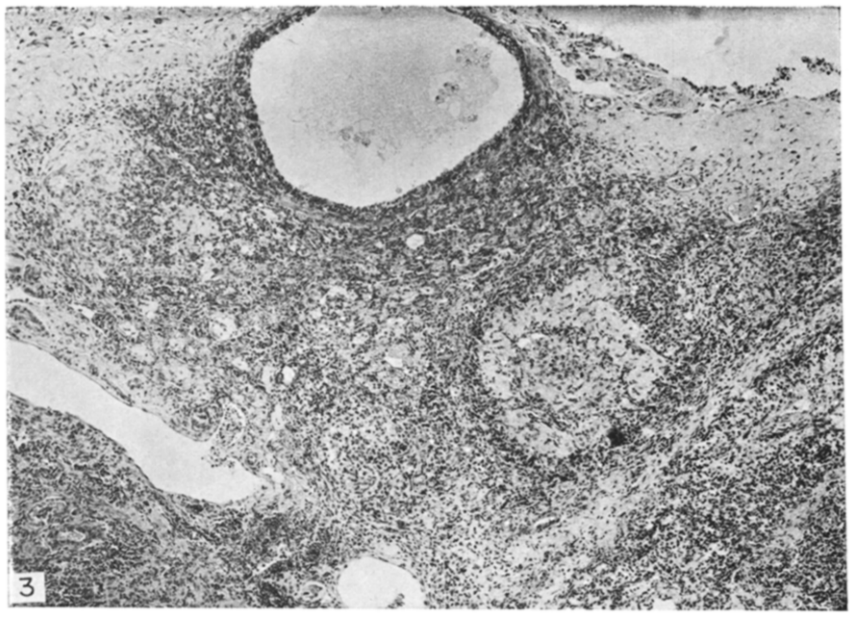


Fig. 3. *Splenic implantation of ovarian tissue with bilateral ovariectomy and a delayed portacaval shunt. Atrophic changes in an implant* 4 *weeks after portacaval shunt. H. & E.* × 120.

Table 1. *Concentrations of LH, FSH and prolactin in the plasma of rats, bilaterally ovariectomized and with implants of ovarian tissue in the spleen (Group Ib)*

Days after implantation	Number of Rats	ng/ml LH	FSH	PRL
0	16	89 (69–248)	258 (160–850)	11 (1–107)
1	4	86 (60–103)	†1570 (775–1420)	49 (18–53)
7	4	*153 (90–175)	†1830 (1420–>2500)	2 (1–9)
9	4	*155 (90–378)	†2100 (1470–2130)	6 (1–72)
20	4	†488 (200–595)	†>2500 (1385–>2500)	1 (1–1)

Blood samples taken under ether anaesthesia from 16 rats before operation and by decapitation of sets of 4 rats at 1, 7, 9 or 20 days after operation. LH, luteinizing hormone, (NIAMD Rat LH-RP-1); FSH, follicle stimulating hormone (NIAMD Rat FSH-RP-1); PRL, prolactin (NIAMD Rat prolactin-RP-1). *P*(vs 0 days); *<0·05; †<0·002; (Mann-Whitney U test). Median and range are shown for each set of values.

Table 2. *Concentrations of LH, FSH and prolactin in the plasma of* 5 *rats, bilaterally ovariectomized and with implants of ovarian tissue in the spleen (Group Ic)*

Days after implantation	Number of rats	ng/ml LH	FSH	PRL
0	5	70 (65–75)	335 (285–510)	33 (13–37)
20	5	†900 (685–1010)	†>2500 (>2500–>2500)	19 (16–21)
35	5	†590 (530–605)	*>2500 (>2500–>2500)	21 (10–45)
49	5	*783 (745–845)	*2500 (>2500–>2500)	24 (20–47)
140	5	†870 (260–1270)	>2500 (210–>2500)	30 (9–102)

Blood samples taken under ether anaesthesia before operation and at 20, 35, 49 and 140 days after operation. LH, luteinizing hormone (NIAMD Rat LH-RP-1); FSH, follicle stimulating hormone (NIAMD Rat FSH-RP-1); PRL prolactin (NIAMD-Rat prolactin-RP-1). *P*(vs 0 days); *<0·016; †<0·008; (Mann-Whitney U test). Median and range are shown for each set of values.

a further 25 days. Plasma hormone levels in unilaterally castrated animals possessing ovarian implants in the spleen were similar to those seen in intact animals (Table 3). Removal of the remaining ovary produced a rapid increase in plasma FSH levels and a slower increase in LH levels. There was a slight fall in plasma concentrations of prolactin.

Portacaval shunt followed by splenic implantation of ovarian tissue and bilateral ovariectomy.

In this group (III) it was noted that the implants rapidly atrophied and little more than a scar was visible on the surface of the spleen by the 77th day after implantation.

Histological examination of the implants showed that an initial period of necrosis and

Table 3. Effect of removal of remaining ovary on the concentrations of LH, FSH and prolactin in the plasma of unilaterally ovariectomized rats bearing implants of ovarian tissue in the spleen (Group II)

Days after total ovariectomy	Number of rats	ng/ml		
		LH	FSH	PRL
0	9	43 (15–120)	390 (320–>2500)	7 (4–26)
25	4	†438 (160–720)	†2290 (1690–>2500)	*4 (2–6)

Blood samples taken under ether anaesthesia immediately before and 25 days after total ovariectomy. LH, Luteinizing hormone (NIAMD Rat LH-RP-1); FSH, follicle stimulating hormone (NIAMD Rat FSH-RP-1); PRL, prolactin (NIAMD Rat prolactin-RP-1). *P* (vs 0 days); * < 0·05; † < 0·02; (Mann–Whitney *U* test). Median and range are shown for each set of values.

Table 4. Histological changes in ovarian implants in the spleen of bilaterally ovariectomized rats. Effect of portacaval anastomosis performed at time of ovarian implantation in the spleen

Age of implant at autopsy (days)	Histological findings	
	Bilateral ovariectomy alone	Bilateral ovariectomy and portacaval anastomosis
1–4	Necrosis in follicles and corpora lutea. Acute inflammatory reaction. Occasional surviving follicles.	Necrosis: inflammation.
5–7	Some necrosis: inflammatory reaction resolving. Development of new follicles. Luteinization beginning.	Necrosis: inflammation resolving: new follicles.
8–14	New follicles. Luteinization, some large corpora lutea becoming cystic, haemorrhagic or fibrotic.	Necrosis: inflammation. Some isolated patches of luteal cells.
21–35	Few new follicles. Large corpora lutea with cystic, haemorrhagic or fibrotic reaction. Some compression of corpora lutea.	Necrosis: follicles: patches of luteal cells.
42–77	Large corpora lutea undergoing cystic haemorrhagic or fibrotic reaction.	Follicles.

Table 5. Effect of portacaval shunt (or laparotomy alone) on the size of ovarian implants in the spleen of bilaterally ovariectomized animals (Group IV)

Operation	Number of rats	Weeks after implant	Length (mm)		Width (mm)	
			Before	After	Before	After
P–C shunt	10	20 (15–25)	6·0 (3·7–10·0)	3·5 (1·9–6·3)	5·0 (1·9–10·0)	2·0 (1·2–4·5)
Laparotomy (Controls)	4	18 (15–18)	4·6 (3·4–5·5)	5·7 (4·5–5·9)	3·5 (0·5–4·0)	4·0 (2·0–4·4)

Animals killed 4 weeks after operation. *P* (shunt vs controls). < 0·02 (before/after for length); *P* (shunt vs controls). < 0·002 (before/after for width).

(Mann–Whitney *U* test with correction for varying ages of implants). Median and range are shown for each set of values.

associated inflammatory reaction occurred, similar to that seen in Group I, but the inflammatory change appeared to last somewhat longer (Table 4). Although follicular development was evident, the follicles did not enlarge nor did they progress to luteal formation. In some animals, isolated groups of luteal cells were noted within the stroma from the 14th day onwards (Fig. 2). There was no further development within the implant up to the 77th post-operative day.

Single blood samples taken from individual animals at 2–13 days after shunt and implant revealed no increase in plasma level of LH and FSH.

Splenic implantation of ovarian tissue with bilateral ovariectomy and a delayed portacaval shunt

No adhesions were observed between the spleen and parietal peritoneum in any of the animals at the time of laparotomy, portacaval shunting or at autopsy. The dimensions of the splenic implants (measured at autopsy) were significantly smaller than those of the same implants measured at the time of portacaval anastomosis (Table 5).

Histologically, the implants from those animals which had undergone portacaval shunts were found to be greatly different from animals in Group I. In general they consisted of patches of ovarian stroma and the remaining follicles were found to be atrophic and cystic. The corpora lutea were few in number, small and centrally scarred (Fig. 3). In one animal, luteal cells appeared to be diffused throughout the ovarian stroma. Control animals which had undergone laparotomy (without portacaval shunting) bore implants which had a histological appearance similar to that observed in Group I.

DISCUSSION

In our experiments, ovarian implants in the spleen underwent a series of histological changes culminating in follicle formation and intense luteinization. Similar observations were made by Biskind and Biskind [1] although they observed the development of luteomata in implants after 150 days.

Biological assays have demonstrated an increase in the content of gonadotrophins in the pituitary and blood after ovarian implantation in the spleen [12–14]. In addition, earlier work also provided evidence that FSH levels in the blood rise more rapidly than those of LH [15]. Our results confirm these reports but we were unable to substantiate the suggestion that raised prolactin levels may also contribute to the luteinization that occurs [16].

Ovarian implants did not grow in unilaterally overiectomized animals until the remaining ovary was removed. This finding was related to an increase in plasma levels of LH and FSH. On the other hand splenic implants failed to grow in animals with a portacaval shunt and there was no change in plasma levels of LH and FSH. Therefore there is an excellent correlation between growth of an implant and plasma concentrations of FSH and LH. It is of interest in this respect that injections of pregnant mare serum gonadotrophin (which possesses LH and FSH activity) accelerate the growth of ovarian implants whereas the administration of ovine prolactin has no effect [9].

Tumour development was not observed in our studies and may depend on increases in prolactin secretion. Injection of ovine prolactin does not stimulate luteoma formation in rats [9], but this procedure may induce the formation of antibodies to prolactin which would block its biological action. It remains to be determined whether induction of raised plasma concentrations will enhance the formation of tumours in implanted ovaries in an analogous manner to that of mammary tumours induced by polycyclic hydrocarbons.

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REFERENCES

1. M. S. Biskind and G. R. Biskind. Development of tumours in the rat ovary after transplantation into the spleen. *Proc. Soc. exp. Biol. (N.Y.)* **55,** 176 (1944).
2. B. M. Peckham, R. R. Greene and M. E. Jeffries. Granulosa cell tumours in female rats and rabbits. *Science* **107,** 319 (1948).
3. B. M. Peckham and R. R. Greene. Experimentally produced granulosa cell tumours in rats. *Cancer Res.* **12,** 25 (1952).

4. B. M. Peckham and R. R. Greene. Experimentally produced granulosa cell tumours in rabbits. *Cancer Res.* **12,** 654 (1952).
5. A. Lipschutz, H. Ponce de Leon, E. Woywood and O. Gay. Intrasplenic ovarian graft in the guinea pig and the problem of neoplastic reactions of the graft. *Rev. Canad. Biol.* **5,** 181 (1946).
6. M. H. Li and W. U. Gardner. Tumours in intrasplenic ovarian transplants in castrated mice. *Science* **105,** 13 (1947).
7. M. H. Li and W. U. Gardner. Further studies on the pathogenesis of ovarian tumours in mice. *Cancer Res.* **9,** 35 (1949).
8. W. U. Gardner. Development and growth of tumours in ovaries transplanted into the spleen. *Cancer Res.* **15,** 109 (1955).
9. G. R. Biskind, D. E. Bernstein and S. M. Gospe. The effect of exogenous gonadotrophins on the development of experimental ovarian tumours in rats. *Cancer Res.* **13,** 216 (1953).
10. H. Bismuth, J. P. Benhamou and J. Lastaste. Technique of portacaval anastomoses in the rat. *Presse med.* **71,** 1859 (1963).
11. V. Danutra, M. E. Harper, A. R. Boyns, E. N. Cole, B. G. Brownsey and K. Griffiths. The effect of certain stilboestrol analogues on plasma prolactin and testosterone in the rat. *J. Endocr.* **57,** 207 (1973).
12. E. C. Jungck, C. G. Heller and W. O. Nelson. Regulation of pituitary gonadotrophic secretion. Inhibition by oestrogen or inactivation by the ovaries. *Proc. Soc. exp. Biol (N.Y.)* **65,** 148 (1947).
13. O. J. Miller and C. A. Pfeiffer. Demonstration of increased gonadotrophic hormone production in castrated mice with intrasplenic ovarian grafts. *Proc. Soc. exp. Biol (N.Y.)* **75,** 178 (1950).
14. W. E. Achilles and S. H. Sturgis. The effect of the intrasplenic ovarian graft on pituitary gonadotrophins. *Endocrinology* **49,** 720 (1951).
15. C. Biddulph and R. K. Meyer. Gonadotrophic hormone secretion in immature hypophysectomised parabiotic rats. *Proc. Soc. exp. Biol. (N.Y.)* **63,** 42 (1946).
16. E. B. Astwood. Regulation of corpus luteum function by hypophyseal luteotrophin. *Endocrinology* **28,** 309 (1941).

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Immunotherapy with Allogeneic Hyperimmune Peritoneal Cells in a Murine Lymphoma System

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Abstract—*Allogeneic C57BL hyperimmune peritoneal cells are used for eradication of lymphoma cells in the peritoneal cavity of syngeneic DBA/2 mice. 2×10^6 SL2 cells injected i.p. 2 hr before treatment with hyperimmune peritoneal cells could be eradicated successfully. The optimal time for collection of the hyperimmune peritoneal cells is 5 days after the last immunization.*

The number of immunizations and the various time intervals between them have only minor influence on the therapeutic potency of the hyperimmune peritoneal cells.

INTRODUCTION

TREATMENT of human tumours by immunotherapy is being done for over a decade. There is, however still much uncertainty about the optimal method of immunization against tumours even in experimental models with animals [1]. Factors involved are e.g. the number of immunizations, time intervals between immunizations, the number of cells used, the site of immunization etc. Consequently immunization is often a process of scientific "hit or miss".

In a previous paper we described the application of immunotherapy with allogeneic immune peritoneal cells in DBA/2 mice bearing the syngeneic SL2 lymphoma in the peritoneal cavity [2]. In this paper we report the application of hyperimmune allogeneic cells for therapy in the same system.

MATERIALS AND METHODS

Mice

Pure bred (a) C57BL, (b) DBA/2, and (c) CBA mice, 6–8 weeks old, were used as (a) a source of hyperimmune peritoneal cells, (b) for growth of the syngeneic ascitic lymphoma SL2, SL2 bearing mice to be cured, and (c) for growth of the syngeneic ascitic lymphoma TLC5.

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Lymphomas

The DBA/2 lymphoma SL2, which arose spontaneously as an ascitic tumour was maintained by weekly i.p. passage. The CBA lymphoma TLC5 was acquired through Dr. P. Alexander and was maintained in CBA mice by weekly i.p. passage.

Hyperimmunization

C57BL mice were hyperimmunized with 3 or 4 i.p. injections of 10^7 DBA/2 derived SL2 lymphoma cells at various time intervals. The following immunization schedules were used:

Schedule 1: Immunizations at day 0, 9 and 18.

Schedule 2: Immunizations at day 0, 9, 18 and 27.

Schedule 3: Immunizations at day 0, 10, 17 and 22.

Control C57BL mice were immunized according to schedule 3 with (a) 10^7 TLC5 lymphoma cells or (b) 10^7 DBA/2 liver cells.

Hyperimmune peritoneal cells were harvested at various times after the last immunization.

Collection of peritoneal cells

Allogeneic hyperimmune and non-immune peritoneal cells were obtained by washing the peritoneal cavity with 5 ml of Fischer's medium (3). Cells were centrifuged and resuspended in Fischer's medium.

Collection of spleen cells

The spleen was dissected and the cells were rinsed out from the spleen by injecting 5 ml of Fischer's medium.

Collection of lymph node cells

Lymph node cells were obtained from the lymphonodi mandibulares present beside the thyroid gland.

Cells used for therapy

2×10^6 hyperimmune C57BL macrophages contaminated with lymphocytes (2) suspended in 1 ml of Fischer's medium were injected i.p. into DBA/2 mice 2 hr following challenge with SL2 cells.

Cells used as controls

(a) 2×10^6 hyperimmune C57BL peritoneal lymphocytes freed of macrophages by exposing the exudate two times to glass for 30 min at 37°C. The lymphocyte suspensions were contaminated with less than 4% macrophages.

(b) 2×10^6 hyperimmune C57BL peritoneal macrophages were freed of lymphocytes by injecting the hyperimmune C57BL mice with 0·3 ml Anti-Lymphocyte Serum (ALS)* 45 minutes before collection of the exudate. Macrophages were contaminated with less than 7% lymphocytes.

(c) 2×10^6 non-immune allogeneic C57Bl peritoneal macrophages, contaminated with lymphocytes.

(d) 2×10^6 hyperimmune (v.s. SL2) lymph-node cells, obtained from the lymphonodi mandibulares.

(e) 2×10^6 allogeneic peritoneal macrophages contaminated with lymphocytes, from mice hyperimmunized against the CBA derived lymphoma TLC5.

(f) 4×10^6 C57BL hyperimmune (v.s. SL2) lymphoid cells of the spleen.

(g) 4×10^6 C57BL nonimmune lymphoid cells of the spleen.

(h) lymphocytes contaminating 2×10^6 hyperimmune peritoneal macrophages.

Hyperimmune control cells were obtained from mice immunized according to schedule 2, and collected 5 days after the last immunization.

*ALS: Anti-Lymphocyte Serum (horse antimouse) was donated by the Radiobiological Institute, TNO, Rijswijk.

Microscopical examination

Tissue sections for microscopical examination were stained with Haematoxylin-eosin.

RESULTS

C57BL mice were immunized i.p. 3 or 4 times with 10^7 SL2 lymphoma cells at various time intervals (see also Materials and Methods). 2×10^6 hyperimmune peritoneal macrophages contaminated with lymphocytes were collected 2, 5, 9 or 16 days after the last immunization.

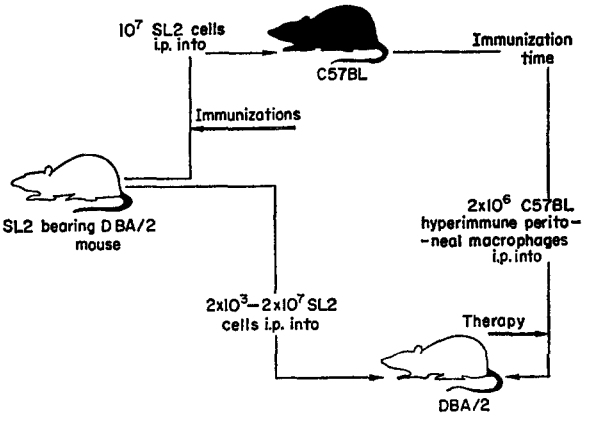


Fig. 1. Scheme above outlines i.p. hyperimmunization of allogeneic C57BL mice with 10^7 DBA/2 derived SL2 lymphoma cells and treatment of DBA/2 mice with 2×10^6 hyperimmune peritoneal macrophages 2 hr after i.p. injection of 2×10^3–2×10^7 SL2 cells.

2×10^6 hyperimmune peritoneal macrophages were injected i.p. into DBA/2 mice 2 hr after 2×10^3–2×10^7 DBA/2 derived SL2 lymphoma cells (Fig. 1). The results of the therapy with hyperimmune peritoneal cells obtained with the immunization schedules 1, 2 and 3 are shown in Tables 1, 2 and 3 respectively. These results are summarized in Fig. 2.

Comparison of these results shows that the therapeutic value of hyperimmune peritoneal cells is optimal 5 days after the last immunization (Fig. 2) independant of the immunization schedule.

With one immunization schedule (Table 3) the result seems optimal if the peritoneal cells are collected 2 days after the last immunization (Fig. 2); however, careful analysis of the figures in Table 3 shows that the absolute survival time is better if hyperimmune peritoneal cells are collected 5 days after the last immunization.

In one experiment one out of five mice survived an i.p. injection of 2×10^7 SL2 cells for more than 35 days (Table 2).

Table 1. *The effect of i.p. injection of C57BL allogeneic hyperimmune peritoneal cells (immunization schedule* 1) *into DBA/2 mice* 2 *hr following challenge with SL2 lymphoma cells*

No. of SL2 cells	No. of survivors at 35 days (real survival time in parentheses) following treatment with hyperimmune peritoneal cells*				
	Collected 2 days after the last immunization	Collected 5 days after the last immunization	Collected 9 days after the last immunization	Collected 16 days after the last immunization	Medium only†
2×10^3	1/5 (38)	5/5 (5>120)‡	3/5 (43, 2>120)‡	2/5 (37, 51)	0/5 (17·6±0·8)
2×10^4	1/5 (35)	3/5 (37, 2>120)‡	1/5 (41)	1/5 (1>150)‡	0/5 (16·0±0·9)
2×10^5	2/5 (35, 1>70)‡	4/5 (35, 39, 40, 1>120)‡	1/5 (41)	1/5 (1>150)‡	0/5 (14·3±1·1)
2×10^6	0/5 (26·6±4·6)	2/5 (37, 51)	1/5 (40)	0/5 (14·0±0.0)	0/5 (11·0±1·3)
2×10^7	0/5 (18·2±5·1)	0/5 (17·0±0·0)	0/5 (16·6±3·7)	0/5 (12·0±1·0)	0/5 (10·1±1·2)
Total number of survivors at 35 days	4	14	6	4	0

*Hyperimmune: C57BL allogeneic peritoneal cells from mice immunized at day 0, 9, 18 and collected 2, 5, 9, or 16 days after the last immunization. 2×10^6 hyperimmune macrophages (plus lymphocytes) were injected i.p. into DBA/2 mice 2 hr following challenge with SL2 cells.

†Mice were injected i.p. with 1 ml of Fischer's medium 2 hr following challenge with SL2 cells.

‡Number of days of survival at the moment this paper was written.

Table 2. *Effect of i.p. injection of C57BL allogeneic hyperimmune peritoneal cells (immunization schedule* 2) *into DBA/2 mice* 2 *hr following challenge with SL2 cells*

No. of SL2 cells	No. of survivors at 35 days (real survival time in parentheses) following treatment with hyperimmune peritoneal cells*				
	Collected 2 days after the last immunization	Collected 5 days after the last immunization	Collected 9 days after the last immunization	Collected 16 days after the last immunization	Medium only†
2×10^3	3/5 (35, 2>70)‡	4/5 (41, 3>80)‡	3/5 (38, 39, 48)	1/5 (48)	0/5 (16·8±0·8)
2×10^4	1/5 (1>70)‡	5/5 (65, 4>80)‡	1/5 (39)	0/5 (25·4±2·5)	0/5 (14·8±0·8)
2×10^5	0/5 (24·0±3·2)	3/5 (45, 2>80)‡	1/5 (1>82)‡	1/5 (35)	0/5 (13·5±0·4)
2×10^6	0/5 (23·0±3·4)	1/5 (36)	0/5 (14·4±1·7)	0/5 (13·6±0·5)	0/5 (11·5±0·8)
2×10^7	0/5 (17·0±2·4)	1/5 (44)	0/5 (11·0±1·1)	0/5 (14·0±1·1)	0/5 (10·3±2·0)
Total number of survivors at 35 days	4	14	5	2	0

*Hyperimmune: C57BL allogeneic peritoneal cells from mice immunized at day 0, 9, 18, and 27 and collected 2, 5, 9 or 16 days after the last immunization. 2×10^6 hyperimmune macrophages (plus lymphocytes) were injected i.p. into DBA/2 mice 2 hr following challenge with SL2 cells.

†Mice were injected i.p. with 1 ml of Fischer's medium 2 hr following challenge with SL2 cells.

‡Number of days of survival at the moment this paper was written.

Table 3. Effect of i.p. injection of C57BL allogeneic hyperimmune peritoneal cells (immunization schedule 3) into DBA/2 mice 2 hr following challenge with SL2 cells

No. of SL2 cells	No. of survivors at 35 days (real survival time in parentheses) following treatment with hyperimmune peritoneal cells*				
	Collected 2 days after the last immunization	Collected 5 days after the last immunization	Collected 9 days after the last immunization	Collected 16 days after the last immunization	Medium only†
2×10^3	4/5 (37, 69, 67, 1>300)‡	4/5 (35, 37, 39, 41)	2/5 (2>80)‡	1/5 (39)	0/5 (16·8 ± 1·9)
2×10^4	3/5 (38, 50, 1>300)‡	3/5 (41, 2>100)‡	0/5 (25·4 ± 2·0)	1/5 (1>70)‡	0/5 (16·0 ± 2·1)
2×10^5	4/5 (37, 38, 42, 62)	3/5 (3>100)‡	1/5 (1>80)‡	0/5 (20·6 ± 6·3)	0/5 (15·4 ± 2·3)
2×10^6	2/5 (37, 56)	2/5 (37, 1>100)‡	0/5 (21·3 ± 4·9)	0/5 (14·0 ± 2·6)	0/5 (12·3 ± 1·3)
2×10^7	0/5 (13·2 ± 4·0)	0/5 (14·0 ± 2·5)	0/5 (11·0 ± 0·1)	0/5 (9·6 ± 0·5)	0/5 (10·1 ± 0·6)

*Hyperimmune: C57BL allogeneic peritoneal cells from mice immunized at day 0, 10, 17, and 22 and collected 2, 5, 9, or 16 days after the last immunization. 2×10^6 hyperimmune macrophages (plus lymphocytes) were injected i.p. into DBA/2 mice 2 hr following challenge with SL2 cells.

†Mice were injected with 1 ml of Fischer's medium 2 hr following challenge with SL2 cells.

‡Number of days of survival at the moment this paper was written.

Table 4. Effect of i.p. injection of various control cell suspensions into DBA/2 mice 2 hr after challenge with 2×10^5 SL2 cells

Control cell suspensions	Number of survivors at 35 days (real survival time in parentheses)
2×10^6 hyperimmune peritoneal lymphocytes	0/5 (27·2 ± 5·2)
Number of lymphocytes contaminating 2×10^6 hyperimmune peritoneal macrophages.	0/5 (14·8 ± 1·5)
2×10^6 hyperimmune peritoneal macrophages	0/5 (15·7 ± 1·7)
4×10^6 hyperimmune lymphoid cells of the spleen	1/5 (1>80)*
4×10^6 non-immune lymphoid cells of the spleen	0/5 (14·0 ± 1·1)
2×10^6 hyperimmune lymph node cells	0/5 (14·2 ± 1·3)
2×10^6 non-immune lymph node cells	0/5 (14·2 ± 0·4)
2×10^6 hyperimmune (vs TLC5) peritoneal macrophages, contaminated with lymphocytes	0/5 (14·0 ± 0·0)
Fischer's medium only†	0/5 (12·3 ± 0·5)

*Time of survival at the moment this paper was written.

†Mice were injected with 1 ml Fischer's medium two hr after challenge with SL2 cells.

In the other 14 mice challenged with 2×10^7 SL2 cells a considerable prolongation of the survival time was obtained on treatment with hyperimmune cells collected 5 days after the last immunization (Tables 1 and 3).

Experiments in which the control suspensions were tested showed that cells used for therapy did always better (Table 4). Only when 4×10^6 allogeneic hyperimmune (v.s. SL2) C57BL spleen cells are injected (2 hr after challenge with 2×10^5 SL2 cells) 1 out of 5 mice survived for more than 35 days. This experiment also showed that the reaction is specific: hyperimmune macrophages (contaminated with lymphocytes) from mice hyperimmunized against the CBA derived TLC5 lymphoma were not capable, to eradicate SL2 lymphoma cells.

Peritoneal exudate cells from hyperimmune C57BL mice did not give rise to any deleterious

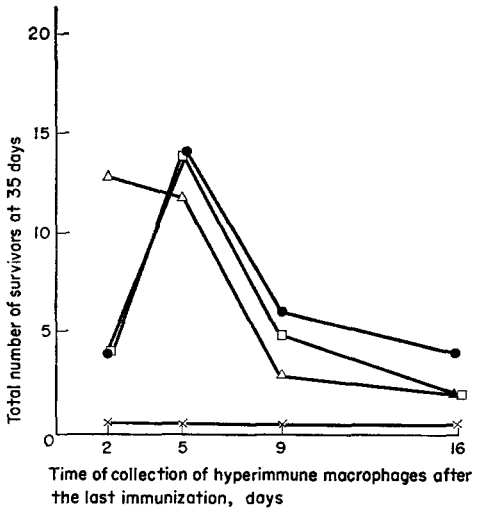


Fig. 2. Influence of the time of collection of the allogeneic hyperimmune peritoneal macrophages (plus lymphocytes) on the total number of survivors at 35 days, if 2×10^6 hyperimmune peritoneal macrophages (plus lymphocytes) are injected i.p. into DBA/2 mice 2 hr following challenge with 2×10^3–2×10^7 SL2 cells.

●—● *immunized at day* 0, 9, *and* 18,
△—△ *immunized at day* 1, 10, 17, *and* 22,
□—□ *immunized at day* 0, 9, 18 *and* 27.
×—× *controls injected i.p. with 1 ml of Fischer's medium, 2 hr following challenge with SL2*

effect in DBA/2 mice as was shown by microscopical examination of haematoxylin–eosin stained tissue sections of gut, liver, spleen, heart, kidney, lung and muscles. Similar results were obtained with non-immune C57BL peritoneal macrophages [2].

DISCUSSION

Allogeneic immune peritoneal macrophages are cytotoxic to tumor cells *in vitro* [4–11] as well as *in vivo* [12–16]. There are also reports about cytotoxicity of immune lymphocytes [16] and of immune or hyperimmune spleen cells [16–18] in *in vitro* systems. Resistance to allograft mouse tumours can be transferred by peritoneal cell suspensions consisting largely or entirely of macrophages [2, 12–15].

Our results show that allogeneic hyperimmune lymphoid cells from the spleen are capable to eradicate lymphoma cells *in vivo* even at a ratio of spleen cells over lymphoma cells of 20:1 (Table 4). Brunner *et al.* [16, 17] and Alexander *et al.* [18] have shown that hyperimmune lymphoid cells are capable to eradicate tumour cells at a ratio lymphoid cells over tumour cells of 100:1 and 200:1 respectively.

Our experiments further show that both hyperimmune lymphocytes and hyperimmune macrophages are necessary to get optimal results. 2×10^6 hyperimmune macrophages or lymphocytes give only a survival time of $15{\cdot}7 \pm 1{\cdot}7$ and $27{\cdot}2 \pm 5{\cdot}2$ days respectively, whereas controls injected with medium died $12{\cdot}3 \pm 0{\cdot}5$ days following a challenge with 2×10^5 SL2 cells (Table 4).

Thus, 2×10^6 purified hyperimmune peritoneal macrophages or 2×10^6 purified hyperimmune peritoneal lymphocytes can not cause a survival of animals injected with 2×10^5 SL2 cells for more than 35 days. However, 2×10^6 hyperimmune macrophages plus contaminating hyperimmune lymphocytes can give a survival time of more than 35 days in five out of fifteen mice injected with 2×10^6 SL2 cells and in one mouse injected with even 2×10^7 SL2 cells.

This suggests the presence of more interactions between macrophages and lymphocytes than simply e.g. fascilitation of antibody production by macrophages by retaining and processing antigen [19–22].

Contacts between target cells, macrophages and lymphocytes seem to be essential for eradication of tumour cells. It is unknown yet to what extent the production of a Specific Macrophage Arming Factor [SMAF, 23–26] by immune lymphocytes is involved in this reaction.

Optimal cytotoxicity of *hyperimmune* peritoneal cells is found 5 days after the last immunization. These cells have a stronger therapeutic potency than *immune* peritoneal cells harvested at the optimal time for cytotoxicity [2, 15].

Experiments in which tumour bearing mice are injected repeatedly with hyperimmune peritoneal cells collected at 5 days after the last immunization are in progress.

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REFERENCES

1. W. den Otter, E. A. Runhaar and C. A. Ruitenbeek, Concomitant immunity in a murine syngeneic lymphoma system. *J. int. Res. Comm.* **1**, No. 3, 24 (1973).
2. H. F. J. Dullens and W. den Otter, Therapy with allogeneic immune peritoneal cells. (Submitted for publication).

3. G. A. Fischer, Studies of the culture of leukemia *in vitro*. *Ann. N.Y. Acad. Sci.* **76,** 673 (1958).
4. G. A. Granger and R. S. Weiser, Homograft target cells: specific destruction *in vitro* by contact with immune macrophages. *Science* **145,** 1427 (1964).
5. G. A. Granger and R. S. Weiser, Homograft target cells: specific destruction *in vitro* by immune macrophages. *Science* **151,** 97 (1966).
6. W. B. Pincus, Cell-free cytotoxic fluids from tuberculin treated guinea pigs. *J. reticuloendoth. Soc.* **4,** 140 (1967).
7. E. R. Heise and R. S. Weiser, Factors in delayed sensitivity: lymphocyte and macrophage cytotoxins in the tuberculin reaction. *J. Immunol.* **103,** 570 (1969).
8. W. E. Hoy and D. S. Nelson, Studies on cytophylic antibodies—V. Alloantibodies cytophylic for mouse macrophages. *Austr. J. exp. biol. med. Sci.* **47,** 525 (1969).
9. K. L. McIvor and R. S. Weiser, Mechanisms of target cell destruction by alloimmune peritoneal macrophages. I. The effect of treatment with sodium fluoride. *Immunology* **20,** 307 (1971).
10. W. den Otter, R. Evans and P. Alexander, Cytotoxicity of murine peritoneal macrophages in tumour allograft immunity. *Transplantation* **14,** 220 (1972).
11. M. L. Lohmann-Matthes, H. Schipper and H. Fischer, Macrophage mediated cytotoxicity against allogeneic target cells *in vitro*. *Europ. J. Immunol.* **2,** 45 (1972).
12. P. Baker, R. S. Weiser, J. Jutila, C. A. Evans and R. J. Blandau, Mechanisms of tumour homograft rejection: the behaviour of Sarcoma I ascites tumour in the A/Jax and the C57BL/6K Mouse. *Ann. N.Y. Acad. Sci.* **101,** 46 (1962).
13. D. B. Amos, In *Mechanism of Cell and Tissue Damage Production by Immune Reactions. II.* International Symposium on Immunopathology; (Edited by P. Grabar and P. Miescher) Schwabe, Basel (1962).
14. B. Bennett, Specific suppression of tumor growth by isolated peritoneal macrophages from immunized mice. *J. Immunol.* **95,** 656 (1965).
15. H. F. J. Dullens and W. den Otter, Eradication of lymphoma cells with allogeneic immune peritoneal cells. *Experientia* **29,** 479 (1973).
16. K. T. Brunner, J. Mauel, H. Rudolf and B. Chapuis. Studies of allograft immunity in mice. I. Induction, development and *in vitro* assay of cellular immunity. *Immunology* **18,** 501 (1970).
17. K. T. Brnner, J. Mauel, J. C. Cerottini and B. Chapuis. Quantitative Assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology* **14,** 181, (1968).
18. P. Alexander, D. I. Connell and Z. B. Mikulska. Treatment of a murine leukemia with spleen cells or sera from allogeneic mice immunized against the tumor. *Cancer Res.* **26,** 1508 (1966).
19. R. E. Franzl and P. D. McMaster, The Primary immune response. I. The enhancement and suppression of hemolysin production by a bacterial endotoxin. *J. exp. Med.* **127,** 1087 (1968).
20. R. E. Franzl and P. D. McMaster, The primary immune response in mice. II. Cellular responses of lymphoid tissue accompanying the enhancement or complete suppression of antibody formation by a bacterial endotoxin. *J. exp. Med.* **127,** 1109 (1968).
21. A. E. Stuart and A. E. Davidson, Effect of simple lipids on antibody after injection of foreign red blood cells. *J. Path. Bact.* **87,** 305 (1964).
22. G. J. Thorbecke and B. Benacerraf, The reticulo-endothelial system and immunological phenomena. *Pro. Allergy* **6,** 559 (1962).
23. R. Evans and P. Alexander, Rendering macrophages specifically cytotoxic by a factor released from immune lymphoid cells. *Transplantation* **12,** 227 (1971).

24. R. EVANS, G. K. GRANT, H. COX, K. STEELE and P. ALEXANDER, Thymus derived lymphocytes produce an immunologically specific macrophage arming factor. *J. exp. Med.* **136,** 1318 (1972).
25. E. PELS and W. DEN OTTER, A cytophylic factor from challenged immune peritoneal lymphocytes renders macrophages specifically cytotoxic. *J. int. Res. Comm.* **1,** No. 2, 28 (1973).
26. E. PELS and W. DEN OTTER, A cytophylic factor from challenged immune peritoneal lymphocytes renders macrophages specifically cytotoxic. (Submitted for publication.)

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Viability of Tumor Cells in Mechanically and Enzymatically Prepared Tissue Suspensions*

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Abstract—*Single cell suspensions were prepared by mechanical disruption or by trypsin or collagenase digestion of mouse livers containing about* 10^7 *viable* ^{125}I-*iododeoxyuridine labeled L*1210 *cells. Tumor cell losses during tissue disruption were evaluated by monitoring the fractional recovery of* ^{125}I *in the final organ brei. The viability of the recovered L*1210 *cells was tested by recording the rate of* ^{125}I *excretion following transplantation of the liver suspensions into new, nonradioactive hosts.*

Marked toxic effects were observed with all 3 *organ dissociation techniques. Fractional cell recovery was* 91% *after mechanical grinding,* 22% *after trypsin digestion and* 43% *after collagenase digestion. The biological viability of those L*1210 *cells which were recovered in the final organ suspension was* 5–30% *in mechanically prepared tissue suspensions,* 5–10% *in trypsin suspensions, and* 25–35% *in collagenase suspensions. Thus, the combined viable cell yield (cell recovery* × *cell viability) was* 5–30% *after mechanical grinding,* 1–2% *after trypsin incubation, and* 10–15% *after collagenase incubation.*

Viability indices obtained with vital stains such as trypan blue proved unsatisfactory. In some experiments the trypan blue test provided a 90–100% *viability reading when in fact the entire L*1210 *population had been inactivated. It was concluded that viability measures based on vital dyes do not provide a useful estimate of the biological vitality of tumor cells in tissue suspensions.*

INTRODUCTION

QUANTITATIVE evaluation of tumor growth parameters frequently involves the use of artificially produced cells suspensions from solid tumors or from host organs containing disseminated tumor cells [1–4]. Suspensions from such tissues are usually produced either by mechanical disruption of the tissue [1–6] or by enzymatic digestion of the intercellular substances [1, 2, 7, 8]. Both techniques involve drastic maltreatment of the neoplastic cells. The resulting reduction in tumor cell viability, along with possible cell losses during the preparation of the tumor suspension, could lead to serious distortion in the experimental data.

Since the exact number of cells present in the original solid tissue is usually not known, it is difficult to ascertain the precise magnitude of cell loss sustained during organ disruption. Furthermore, existing techniques do not permit accurate evaluation of the viability of those tumor cells which are recovered in the final cell suspension. Most studies on the viability of cells in suspensions are based on results with vital staining tests. However, previous reports have indicated that vital dyes such as trypan blue record only very late stages of cell injury [9].

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These findings along with data presented in this communication suggest that tumor cells may be damaged beyond recovery without actually demonstrating any increase in their stainability with vital dyes.

In our laboratory we have used for several years a different method for determining tumor cell viability, the ^{125}I-iododeoxyuridine (^{125}IUDR) prelabeling assay. This technique basically consists of labeling ascites tumor cells with ^{125}IUDR, an analogue of thymidine, which is incorporated exclusively into the DNA of proliferating cells [10, 11, 12]. If ^{125}IUDR is administered during the log phase of ascites growth, sufficient radioactivity can be introduced into the DNA of the ascites cells to permit continuous evaluation of the rate of tumor cell death following transplantation of these prelabeled cells into new, nonradioactive hosts [13, 14]. Thus, the ^{125}IUDR prelabeling technique should enable us to monitor simultaneously the rate of cell loss during the preparation of cell suspensions and possible adverse effects of the preparation technique on the viability of the tumor population. Outright cell loss during tissue disruption would result in incomplete recovery of ^{125}I in the tumor cell suspension, whereas reduced viability of the cells present in the final suspension would manifest itself in the form of increased ^{125}I loss from secondary recipient hosts.

MATERIALS AND METHODS

L1210 mouse lymphoid leukemia cells [15] were grown in the peritoneal cavity of C3D2F1J female mice by weekly passage of 10^6 cells. On day 4 of tumor growth, the cells were labeled *in vivo* by i.p. administration of ^{125}IUDR (specific activity: 10^5 mCi/mmole; source: Schwarz/Mann, Orangeburg, New York). The total ^{125}IUDR labeling dose was 2 μCi/mouse, subdivided into 4 equal aliquots and injected over a 10 hour labeling period. Using this procedure it was found that there was an uptake of 10–20% of the ^{125}IUDR by the peritoneal L1210 cells and, according to previous studies [14], the labeling index was 98% or higher. The described labeling schedule was closely followed in all experiments to avoid the radiation damage and increased cell death observed after incorporation of high doses of ^{125}I into the DNA of L1210 ascites cells [16].

One day after ^{125}IUDR administration, the labeled tumor cells were aseptically removed from the peritoneal cavity of the labeling mice, pooled, diluted in Earle's balanced salt solution, and centrifuged at 600 RPM for 10 min to remove unincorporated radioiodide. To minimize adverse effects on tumor cell viability, all these procedures were performed at 4°C. When aliquots of the final cell suspension were precipitated with 10% trichloroacetic acid, less than 3% of the radioiodide was found in the supernatant, indicating that more than 97% of the total radioactivity was associated with the DNA of the tumor cells [10, 11].

The prelabled cells were then adjusted to a concentration of 5×10^7 cells/ml and 0·2 ml aliquots (10^7 cells) were injected intravenously into new, nonradioactive hosts. The recipient mice were killed 15–18 hr later and the livers were removed and cleaned of extraneous tissue. The radioactivity present in the livers, usually 20–30% of the i.v. injected ^{125}I activity, was taken as an index of fractional tumor cell migration into this organ [13, 14]. In other words, approximately 20–30% of the inoculated L1210 cells (2–3 million) migrated to the livers of the host mice. Since the population doubling time of L1210 cells after i.v. implantation is about 9–10 hr [17], the 2–3 million L1210 cells which had migrated to the livers must have increased to about 10 million cells during the 15–18 hr time span between tumor inoculation and removal of the leukemic livers.*

Mechanical disruption of the livers was accomplished by placing the whole organ into a simplified stadie-type Latapie tissue grinder. The organ brei was collected aseptically in a 10 ml syringe. After completion of the grinding procedure the tissue grinder was flushed twice with 0·5 ml of Earle's balanced salt solution and the fluid drained from the grinder was mixed with the content of the syringe. This procedure permitted almost complete recovery of the ^{125}I activity present in the original liver. The final organ brei was inoculated i.p. into 5 recipient hosts.

Enzymatic digestion of livers was carried out at room temperature with Ca^{2+}–Mg^{2+}-free collagenase [18] or trypsin solutions (Table 1). The following procedure was used:

1. The leukemic livers were minced with a razor blade on a plexiglas cutting board and the tissue fragments (particle size about 1 mm^3) were incubated for 20 min at 4°C in Ca^{2+}–

*^{125}I excretion following cell death *in vivo* was rapid and essentially complete, i.e. nonspecific retention of ^{125}I activity due to reutilization or phagocytosis of dead labeled cells was minimal. Consequently, the fractional retention of ^{125}I activity within leukemic livers indicates the presence of a corresponding fraction of fully viable L1210 cells (see also Fig. 1 and Table 2).

Table 1. *Dissociation media*

Component	Amount/100 ml
Pancreatin	50 mg
DNAse (2,000 Kunitz units/mg)	2 mg
RNAse (40 Kunitz units/mg)	5 mg
Methyl cellulose	100 mg
Bovine serum albumin	200 mg
Heparin (1,000 units/ml)	1 ml
Calf serum	2 ml
Ca^{2+}–Mg^{2+}-free EBSS	97 ml

This basic solution was stored at a temperature of −90°C. Immediately before the start of organ dissociation either Trypsin–Difco 1:250 (200 mg/100 ml) or Collagenase (200 mg/100 ml) plus Hyaluronidase (100 mg/100 ml) were dissolved in this basic solution. More recent experiments have indicated that reducing the collagenase concentration to 50 mg/100 ml and increasing the incubation time to 90 min gave superior results (higher cell yield and less cell killing).

Mg^{2+}-free Earle's balanced salt solution (CMF solution).

2. After incubation the suspension of tissue fragments was transferred into a constant flow tissue dissociation chamber and the CMF solution was allowed to drain through the filter mounted at the bottom of the chamber.

3. After removal of the CMF solution a constant flow of trypsin or collagenase solution was initiated (2 ml of enzyme solution per min for 45 min at room temperature). Except for the slight turbulence caused by the slow flow of dissociation medium the tissue fragments were not agitated during enzyme incubation. The enzyme solution containing the dissociated liver and leukemia cells was draining continuously through the bottom filter of the chamber and was collected in 100 ml of 4° CMF solution, supplemented with 5% calf serum to reduce enzyme toxicity [19].

4. After 45 min the enzyme solution was replaced by a constant flow of CMF solution (with 5% calf serum) and the remaining small tissue fragments were gradually dissociated by gentle agitation with a Pasteur pipette. This procedure was continued until only little clumps of connective tissue remained in the dissociation chamber. Only trace amounts of ^{125}I activity were found to be associated with these undigested tissue residues.

5. The final cell suspension consisting of ^{125}IUDR labeled L1210 cells plus the dissociated liver cells was centrifuged at 800 rev/min for 10 min and the ^{125}I activity present in the pellet and the supernatant was determined in a well-type crystal scintillation counter. The pellet was washed a second time and resuspended in fresh CMF solution and inoculated i.p. into the peritoneal cavity of 5 recipient mice.

Two different methods, the trypan blue test and the ^{125}IUDR assay, were used to evaluate the viability of the experimental tumor populations. Vital staining tests were performed by mixing a drop of the tumor cell suspension with an equal volume of 0·25% trypan blue. The viability index was determined by counting 300 or more cells in a haemocytometer. The ^{125}I viability assay was based on radioiodide retention data of animals which had been inoculated with enzymatically or mechanically prepared suspensions of livers harboring ^{125}IUDR labeled L1210 cells. For these measurements the recipient animals were placed in a well-type scintillation counter and their whole body ^{125}I activity was determined immediately after inoculation of the organ brei and at daily intervals thereafter. Previous studies have shown that any loss of ^{125}I activity from such mice can be taken as an index of tumor cell death *in vivo* [13, 14].

RESULTS

Organ distribution and viability of i.v. inoculated L1210 cells

Before attempting to use the ^{125}IUDR prelabeling technique for determining the viability of tumor cells in mechanically or enzymatically prepared liver suspensions it was necessary to prove that the livers of leukemic mice are in fact a source of fully viable L1210 cells. Figure 1 shows the whole body retention and the organ distribution of ^{125}I activity during the first 24 hr after i.v. inoculation of ^{125}IUDR labeled L1210 cells. In mice killed immediately after tumor inoculation most of the cells were temporarily trapped in the lungs. However, within 12 hr more than 90% of the inoculated cells had passed through the lungs and had reappeared in other organs of the body, particularly in the liver. Within 12–18 hr a stable anatomical distribution was established which remained essentially unchanged during the remaining 4 days of tumor growth. As pointed out in previous publications [13, 14] there was a steady rate of tumor cell death (20–25%/day) in all recipient organs throughout tumor development, but since the death rate was identical in all organs, the relative organ distribution of the leukemic cells was not affected.

To demonstrate that the retention of ^{125}I in

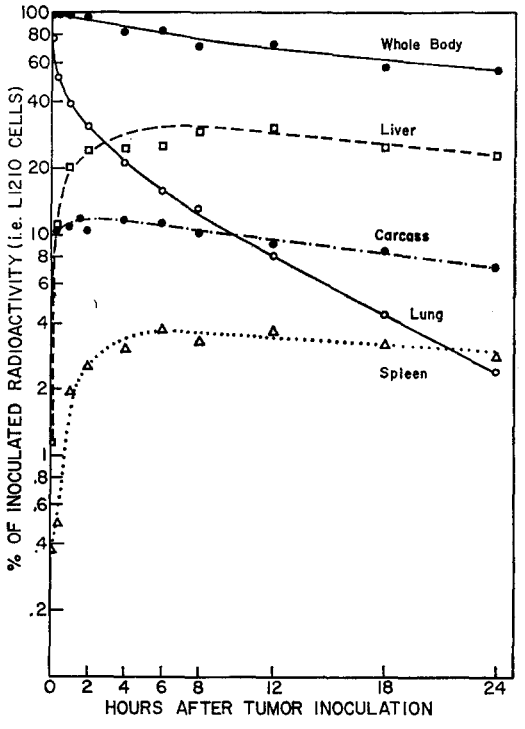


Fig. 1. Anatomical distribution and excretion of ^{125}I activity following i.v. inoculation of 10^7 ^{125}IUDR labeled L1210 cells. Each point represents the average ^{125}I retention of 5 mice, expressed as per cent of the inoculated radioactivity. The variation within each experimental group was less than ± 10% of the mean.

leukemic livers did not represent reutilization or trapping of dead labeled tumor cells, similar i.v. experiments were carried out with mixed inocula of living ^{125}IUDR labeled L1210 ascites cells and lethally irradiated ^{131}IUDR labeled L1210 cells. The ^{131}IUDR labeled cells were irradiated with 5000 rads 1 hr before transplantation into new hosts. A dose of 5,000 rads was chosen because previous studies [20] had shown that such a dose was sufficient to kill all L1210 cells after a lag period of about 24–28 hr. During the lag period the lethally irradiated ^{131}IUDR labeled cells were still able to disseminate into the same organs as the fully viable ^{125}IUDR labeled L1210 cells (Table 2, day 1 data). However, when the irradiated cells died, their ^{131}I activity was rapidly excreted from the body of the recipient mice, whereas large fractions of the ^{125}I activity associated with viable L1210 cells were still present in the same mice on day 4 of tumor growth, the usual day of death of i.v. inoculated leukemic mice (Table 2, day 4 data). Reversing the order of killing, i.e. killing ^{125}IUDR labeled cells and leaving the ^{131}IUDR labeled cells alive gave identical results, except of course that in this case ^{131}I was retained and ^{125} I was excreted (data not shown).

*Table 2. Anatomical distribution of ^{125}I and ^{131}I activity on days 1 and 4 after simultaneous i.v. inoculation of living (^{125}I) and radiation killed (^{131}I) L1210 leukemia cells**

Organ	Live cells (^{125}I) Day 1	Day 4	Radiation-killed cells (^{131}I) Day 1	Day 4
Whole body	64·2	31·4	61·4	3·7
Liver	28·7	14·7	24·1	0·3
Spleen	2·6	1·4	2·5	0·1
Lung	4·4	1·5	6·1	0·1
Rest of body	23·0	10·7	22·1	2·9

*Radioisotope retention values refer to the mean of 5 mice, expressed as per cent of the inoculated counts. The ^{125}I data had to be corrected for a 19·4% overlap of the ^{131}I spectrum in the ^{125}I channel. The S.D. for most organs was approximately ± 10% of the mean.

From these observations it can be concluded that any long-term retention of ^{125}I or ^{131}I within leukemic animals indicates the presence of a corresponding fraction of living, undamaged L1210 leukemia cells, not retention of radioactivity due to reutilization or trapping of dead labeled cells. In other words, since the ^{125}I or ^{131}I activity associated with dying cells is rapidly eliminated from the body of the test mice, the unexcreted fraction of the radioactivity provides an accurate index of fractional tumor cell dissemination and survival *in vivo*.

Tumor cell yield after dissociation of leukemic livers

Tumor cell losses sustained during the preparation of the liver suspensions were evaluated by monitoring the fractional recovery of ^{125}I activity in the final organ brei. Outright cell loss was not an important factor with mechanically prepared tissue suspensions where the cell yield was usually about 90%. However, following enzymatic digestion of livers containing living ^{125}IUDR labeled L1210 cells the recovery rate averaged only about 40–50% for collagenase and 20–30% for trypsin (Table 3). Most of the missing ^{125}I activity was found to be associated with cellular debris present in the supernatant of the enzyme solutions. Since catabolic radioiodide constituted less than 3% of the original liver activity, most of the observed 50–80% loss must have been due to enzyme induced disruption and digestion of intact ^{125}I labeled L1210 cells residing within the liver.

Table 3. *Fractional cell yield after mechanical and enzymatic disruption of leukemic livers* (5 *livers per experimental group*)

Fraction	Mechanical grinding	Trypsin incubation	Collagenase incubation
Original livers counts/min	132,200	141,900	135,800
Liver suspension counts/min	120,300	31,200	58,400
Fractional yield	91%	22%	43%

Tumor cell viability after dissociation of leukemic livers

The biological viability and vitality of the ^{125}IUDR labeled tumor cells present in the final organ brei was tested by inoculating the leukemic liver suspensions into new, non-radioactive hosts. As demonstrated by our studies with mixed inocula of living and dead labeled tumor cells, virtually all of the ^{125}I activity retained in the original intact liver was associated with fully viable L1210 cells. Thus, if the organ dissociation technique did not cause any adverse effect on tumor cell viability, the rate of ^{125}I excretion from mice receiving leukemic liver suspensions should be identical to that of mice inoculated with ^{125}IUDR labeled L1210 ascites cells. Conversely, any tumor cell lethality resulting from tissue dissociation would cause a corresponding decline in the retention of ^{125}I in animals implanted with the leukemic liver suspension.

From the results presented in Fig. 2 it was apparent that the viability of the tumor cells was reduced both in mechanically and in enzymatically prepared tissue suspensions. The damage produced by mechanical organ disruption was comparatively modest in experiments where the organ dissociation was incomplete, i.e. where the organ suspension contained many undissociated liver fragments. However, when the grinding procedure was more thorough there was a severe decline in tumor cell viability and a corresponding increase in the rate of ^{125}I excretion. Using an equation developed in an earlier publication [16], it can be shown by interpolation between live and dead control values that approximately 10% of the tumor cells residing in leukemic livers remained viable after complete organ disruption (Fig. 2). In some cases the survival rate was as low as 5%. In experiments where the grinding procedure was not carried to the single cell state the ^{125}I data indicated survival of up to 30% of the hepatic L1210 population (data not shown).

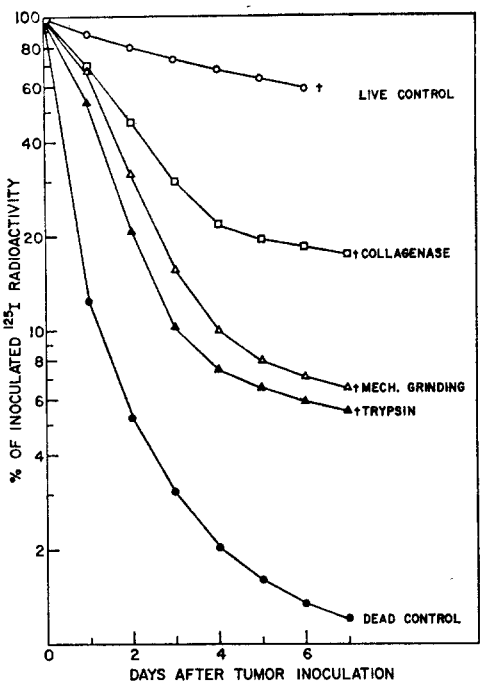


Fig. 2. *Loss of* ^{125}*I from mice inoculated i.p. with mechanically and enzymatically prepared suspensions of leukemic livers. Mice receiving fully viable* (*live control*) *or heat-killed* (*dead control*) *L1210 ascites cells were simultaneously injected with a mechanically prepared cell suspension from non-leukemic livers to simulate the conditions existing in mice receiving leukemic liver suspensions. Each data point represents the average* ^{125}*I retention of* 5 *mice, expressed as per cent of the inoculated radioactivity. The variation within each treatment group was less than* ± 10% *of the mean. The symbol* † *indicates the mean day of death of the experimental animals.*

In contrast, enzyme dissociation gave relatively consistent viability data. Trypsin digestion usually reduced the viability of hepatic L1210 cells to about 5–10% of the live control value. Considering the low cellular recovery rate (22%) after trypsin incubation (Table 2) the combined fractional net yield of viable leukemia cells from trypsin-treated livers was only about 1–2%. Collagenase–Hyaluronidase incubation gave considerably better survival values (25–35%) and a larger cell yield (43%) so the combined net viable yield from collagenase-treated livers was about 10–15%.

Trypan blue test vs ^{125}*IUDR assay*

To evaluate the reliability of the trypan blue test for monitoring cellular viability, samples of ^{125}IUDR labeled L1210 ascites cells were subjected to potentially damaging treatments

Table 4. Viability index of living, irradiated, and heat incubated L1210 ascites cells based on the trypan blue exclusion test. The corresponding ^{125}I assay values are presented in Fig. 4

Treatment of L1210 ascites cells	Trypan Blue Viability Index (%)
Live control	96
Irradiation (10,000 rads)	97
Heat incubation (45°C)	91
Heat incubation (65°C)	0

such as 15 min incubations at 45°C or 65°C and irradiation with 10,000 rads in a ^{137}Cs irradiator. From the trypan blue viability indices listed in Table 4 it would appear that none of these treatments, except incubation at 65°C, had any significant effect on tumor cell viability. However, when the viability of the same population samples was subsequently tested with the ^{125}IUDR assay (Fig. 3) it became apparent that all 3 treatments were equally toxic, i.e. all treatments caused complete biological inactivation of the entire leukemia population and no signs of tumorous growth were ever observed in the recipient hosts.

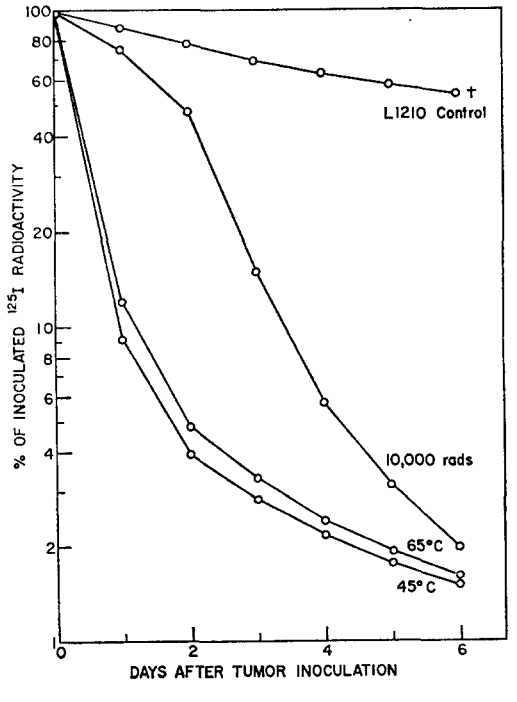


Fig. 3. Loss of ^{125}I from mice inoculated i.p. with living $^{125}IUDR$ labeled L1210 ascites cells, or with cells prekilled by irradiation or heat treatment (legend as in Fig. 2).

From these findings it was obvious that viability data based on trypan blue tests would not provide a useful estimate of the biological vitality of tumor cells in organ suspensions. Nevertheless, occasional trypan blue tests were performed on single cell suspensions of leukemic livers. In all cases, the dye exclusion test gave viability readings which were substantially higher than the readings obtained with the ^{125}IUDR prelabeling assay.

DISCUSSION

Studies with artificially produced tumor cell suspensions suffer from two potential shortcomings:

1. The exact number of tumor cells present in the original tissue is frequently not known which complicates accurate evaluation of the rate of cell loss occurring during the preparation of the cell suspensions.

2. The viability index of those tumor cells which are recovered in the final organ brei can often not be precisely ascertained. Not only is the viability of cells frequently difficult to establish, it ultimately depends on the definition of viability chosen. If the viability index is based on measures of membrane function and/or metabolic activity, then by definition all cells which meet these criteria are "viable" regardless of whether or not they are able to survive after transplantation into new hosts. Such criteria may be satisfactory for certain biochemical studies, but they could prove seriously misleading in quantitative evaluations of tumor growth parameters *in vivo*. A careful distinction between viable and irreversibly damaged cells may also be essential for certain studies on cellular metabolism, because the biochemical processes occurring in a dying cell may be quite different from those of a normal, fully viable cell.

Many investigators seem to rely primarily on the trypan blue test and other vital staining techniques for monitoring the viability of tumor cells in monodispersed tissue suspensions. Not only do such methods not provide any information on the fractional cell yield after tissue disruption, they also have considerable limitations as indices of tumor cell death. The trypan blue test, for example, is so insensitive that it may provide the investigator with a 90–100% viability reading when in fact the entire cell population has been inactivated (Table 4, Fig. 3).

Occasional studies with other "vital stains" such as eosin proved to be similarly disappointing. It appears that such dyes are only

useful for indicating the integrity of the cell membrane at the time of observation, but are of little value for evaluating tumor cell viability in terms of cell survival following transplantation into new hosts. A much more useful appraisal on the state of the tumor population in a tissue suspension can be obtained with the ^{125}IUDR prelabeling technique. This procedure not only indicates the biological viability and long-term survival of tumor cells, it also provides a precise measure of the magnitude of cell loss sustained during the preparation of the tissue suspensions.

Lack of information on either of these parameters could have drastic consequences in quantitative studies on the kinetics of tumor growth. For instance, if an attempt were made to evaluate the number of leukemia cells present in the livers of i.v. inoculated mice, using our liver suspensions as sources for the customary sequential dilution assay [3, 4, 21], the resulting estimate would be grossly inaccurate. In the case of organ suspensions prepared by collagenase incubation the fractional cell yield is approximately 40% (Table 2). Since about 70% of the recovered L1210 cells are inactivated by the trauma associated with collagenase digestion, it follows that the fraction of viable tumor cells actually transplanted into new hosts represents only about 12% of the initial hepatic L1210 population. Consequently, if a sequential dilution test indicated the presence of 5 million tumor cells within the leukemic livers, the actual value would be closer to 40 million. Using trypsin digested liver samples, the correction factor would become astronomical, i.e. an experimental value of 5 million L1210 leukemia cells would have to be adjusted to more than 250 million. With mechanically prepared tissue suspensions the problem would be further complicated by the wide spread in survival values (5–30%). Thus, the presence of 5 million viable tumor cells in a liver suspension could indicate an original population size as small as 15 million or as large as 100 million.

We have recently extended our enzyme dissociation studies to L1210 cells growing in lungs and spleens of i.v. inoculated mice. The results were virtually identical to those obtained with hepatic L1210 cells. Nevertheless, since we have used only one tumor–host system, our findings may not apply to other experimental situations. Even with L1210 cells the response was not completely consistent. Leukemia cells growing in livers, spleens, and lungs were easily damaged by enzyme incubation, whereas L1210 cells growing in ascites form in the peritoneal cavity proved to be more resistant, at least during later stages of tumor development (unpublished observations). Thus, it would not be surprising if different tumor strains displayed large variations in their response to tissue disruption. However, even if the rates of cell loss and cell destruction in other experimental systems are not as dramatic as with organs containing L1210 leukemic cells, our findings suggest that many of the conclusions based on enzymatic and mechanical dispersion techniques may require careful re-evaluation.

REFERENCES

1. B. Boeryd, O. Eriksson, F. Knutson, P. M. Lundin and K. Norrby. On the viability of tumor cells in artificially produced suspensions. *Acta path. microbiol. scand.* **65,** 514 (1965).
2. B. Boeryd, P. M. Lundin and K. Norrby, Tumor growth after intravenous, intraperitoneal and subcutaneous injection of syngeneic monodispersed tumor-cell suspensions. *Europ. J. Cancer* **7,** 557 (1971).
3. H. E. Skipper, F. M. Schabel Jr. and W. S. Wilcox, Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with curability of experimental leukemia. *Cancer Chemother. Rep.* **35,** 1 (1964).
4. H. E. Skipper, F. M. Schabel Jr., W. S. Wilcox, W. R. Laster Jr., M. W. Trader and S. A. Thompson, Experimental evaluation of potential anticancer agents. XVIII. Effects of therapy on viability and rate of proliferation of leukemic cells in various anatomic sites. *Cancer Chemother. Rep.* **47,** 41 (1965).
5. R. Schrek, Studies *in vitro* on the physiology of normal and of cancerous cells. II. The survival and the glycolysis of cells under aerobic and under anaerobic conditions. *Arch. Pathol.* **37,** 319 (1944).
6. J. Vaage, A mechanical technique for obtaining high yields of viable dispersed tumor cells. *Transplantation* **6,** 137 (1968).

7. R. E. MADDEN and D. BURK, Production of viable single cell suspensions from solid tumors. *J. nat. Cancer Inst.* **27,** 841 (1961).
8. K. NORRBY, F. KNUTSON and P. M. LUNDIN, On the single cell state in enzymatically produced tumor cell suspensions. *Exp. Cell Res.* **44,** 421 (1966).
9. D. W. KING, S. R. PAULSON, N. L. PUCKETT and A. T. KREBS, Cell death. IV. The effect of injury on the entrance of vital dye in Ehrlich tumor cells. *Am. J. Pathol.* **35,** 1067 (1959).
10. K. G. HOFER and W. L. HUGHES, Incorporation of iododeoxyuridine-^{125}I into the DNA of L1210 leukemia cells during tumor development. *Cancer Res.* **30,** 236 (1970).
11. W. L. HUGHES, S. L. COMMESFORD, D. GITLIN, R. C. KREUGER, B. SCHUTZE, V. SHAH and P. REILLY, Deoxyribonucleic acid metabolism *in vivo*. I. Cell proliferation and death as measured by incorporation and elimination of iododeoxyuridine. *Fed. Proc.* **23,** 640 (1964).
12. W. H. PRUSOFF, Synthesis and biological activities of iododeoxyuridine, an analog of thymidine. *Biochem. biophys. Acta* **32,** 295 (1959).
13. K. G. HOFER, W. PRENSKY and W. L. HUGHES. Death and metastatic distribution of tumor cells in mice monitored with ^{125}I-iododeoxyuridine. *J. nat. Cancer Inst.* **43,** 763 (1969).
14. K. G. HOFER and M. HOFER, Kinetics of proliferation, migration, and death of L1210 ascites cells. *Cancer Res.* **31,** 402 (1971).
15. L. W. LAW, T. B. DUNN, P. J. BOYLE and J. H. MILLER, Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemia in mice. *J. nat. Cancer Inst.* **10,** 179 (1949).
16. K. G. HOFER and W. L. HUGHES, Radiotoxicity of intranuclear tritium, ^{125}iodine and ^{131}iodine. *Radiation Res.* **47,** 94 (1971).
17. I. WODINSKY, J. SWINIARSKI and C. J. KENSLER. Spleen colony studies of leukemia L1210. I. Growth kinetics of lymphocytic L1210 cells *in vivo* as determined by spleen colony assay. *Cancer Chemother. Rep.* **51,** 415 (1967).
18. T. YAMADA and E. J. AMBROSE, Preparation of cell suspensions from tissues using collagenase and hyaluronidase especially for measuring the electrical charges of surface membranes of mammalian cells. *Exp. Cell Res.* **44,** 634 (1966).
19. R. D. CAHN, H. G. COHN and M. B. CAHN, Cell culture and cloning techniques. In *Methods in Developmental Biology.* (Edited by WILT & WESSELS) pp. 493–530; Y. T. Crowell, New York (1967).
20. K. G. HOFER, Radiation effects on death and migration of tumor cells in mice. *Radiat. Res.* **43,** 663 (1970).
21. H. B. HEWITT and C. W. WILSON, A survival curve for mammalian leukemia cells irradiated *in vivo* (implications for the treatment of mouse leukemia by whole-body irradiation). *Brit. J. Cancer* **13,** 69 (1959).

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Phase 1 Study with Vitamin A Acid Ethylamid in Man*

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Abstract—*Vitamin A Acid Ethylamid (VAAE), a new derivative of vitamin A acid under study in tumor therapy, was given orally in increasing doses to eleven patients to assess its toxicity. As compared with other congeners of vitamin A the side effects occur at higher doses (between 300 and 1800 mg daily). Most common among these are angular cheilitis and pruritic erythematous rashes, both usually tolerable. Two patients with cerebral metastases developed severe headache and dizziness with 300 mg, a complaint reported as well in two other patients with organic brain disease at higher doses. There was no evidence of bone marrow, liver or kidney toxicity. In those patients with measurable tumors no therapeutic effect was detected.*

INTRODUCTION

AMONG the vitamin A series under investigation vitamin A acid (NSC-122758) has been reported to be effective in the therapy of certain premalignant and malignant epithelial lesions. Systemic administration in mice with chemically induced benign and malignant skin tumors resulted in regression of a significant number of cases [1]. In man the topical application of vitamin A acid leads to partial or complete regressions of actinic keratoses and basal cell carcinomas of the skin [2, 3]. The oral administration has a favourable influence on precancerous lesions of the skin and of the mucous membranes of the upper respiratory tract [4], as well as on papillomas of the urinary bladder [5]. The exact mode of action is only partially elucidated. There is evidence that it is involved in the growth and differentiation of ectodermal tissues and that the possible site of action is the lipoprotein membrane of cells and of their organelles, particularly lysosomes [6–8]. It is also thought that the induced changes in membrane permeability e.g. the lysosomal labilization might make tumor cells more vulnerable to alkylating agents. Thus, vitamin A alcohol, was found to enhance the effect of Cytoxan and BCNU in mouse mammary adenocarcinoma [8]. A serious drawback of vitamin A acid, however, is the appearance of side effects at relatively low doses (150–300 mg/day).

This prompted the search for a less toxic congener offering an identical therapeutic effect. In animal experiments vitamin A acid ethylamid (VAAE) was found to be a product offering at least five times less toxicity than vitamin A acid [9].

The LD 50 in the mouse and the rat is $>$ 4000 mg VAAE given orally or intraperitoneally. Chronic toxicity studies (13 weeks' experiment with 250 mg/kg orally q.d. $\times$ 5 days per week) lead to reduced weight increase in young animals, slight anemia, hyperkeratosis of skin, hypotrophy of hair follicles and bone fractures in rats as well as slight weight loss, slight anemia and reduced spermiogenesis in

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*Vitamin A acid ethylamid = ethylamid of all-trans-B-retinoic acid, kindly supplied by Dr. W. Bollag, F. Hoffmann-La Roche Co., Ltd., Basel, Switzerland.

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dogs [9]. 50 mg/kg in rats and 100 mg/kg in the dog during 13 weeks were well tolerated.

The purpose of this study was to establish the tolerance of VAAE in man.

METHODS

Vitamin A acid ethylamid (VAAE) was available in capsules containing 50 and 200 mg of the compound, respectively. It was taken orally t.i.d. in increasing doses until untoward reaction or rapid tumor progression occurred, precluding further treatment. In one instance it was stopped, for unrelated reasons, when a total daily dose of 2000 mg was attained without side effect.

The first 2 patients on study received the product in increments of 50 mg with a starter dose of 50 mg. When the mild toxicity became evident, the subsequent starting dose was set at 200 mg with an increase of 200 mg every four days. After a total daily dose of 1000 mg was attained the 200 mg increases occurred weekly.

PATIENT SELECTION

Eleven patients entered the study between January and August 1971. Ten of them had solid, mostly metastatic, tumors either refractory to radiotherapy, chemotherapy or both, or inoperable and asymptomatic at onset. One chronic non-tumor patient was recovering from a cerebro-vascular accident sustained four months before. Six of the patients were hospitalized in the Basle University Hospital at the beginning of the study and five were followed in the Oncology Out-patient Department.

Three patients had undergone no prior therapy because they were asymptomatic and their tumors inoperable (two bronchogenic carcinomas, one questionable carcinoma of the head of the pancreas). Two patients had been previously treated with one or several courses of chemotherapy; three had undergone surgery followed by radiotherapy, and the remainder surgery, radiotherapy and one or several attempts at chemotherapy. Five patients had masses measurable by X-rays, or grossly, by scitigraphy. The others had either subjective complaints or hardly measurable tumor manifestations.

There were 8 men and 3 women with a mean age of 56 years (range 26–78 years). Of the patients with tumors the medium number of months from diagnosis to treatment with VAAE was 25 months (range 3–91 months). The average duration of treatment with the new compound was 59 days (range 6–276 days).

The cases were weekly evaluated with clinical parameters, hemograms, blood chemistries and radiologic follow up.* The clinical parameters included weight, temperature, general condition, pain, appetite, gastrointestinal, respiratory, neurological and cutaneous symptoms and signs. The blood chemistries comprised bound urea nitrogen, creatinine, uric acid, calcium, potassium, alcaline phosphatase, serum glutamic pyruvic transaminase, bilirubin and total protein.

RESULTS

Nine of the patients developed, at doses between 300 and 1800 mg, clinical signs or symptoms of toxicity requiring discontinuation of the drug. In one there were no signs of toxicity with a total daily intake of 2000 mg, but the tumor progression dictated a change in therapeutic approach. Another patient refused treatment at 800 mg/day for reasons unrelated to toxicity.

Clinical side effects

Ectodermal side effects (angular cheilitis, stomatitis, skin rashes) were seen in 6 patients, in one of whom discontinuation was required (ulcerative stomatitis). In the others the dose was either decreased or topical treatment brought relief. Headache, associated or not with dizziness and/or vomiting, was reported by 4 patients. Two of them had documented cerebral metastases (symptoms appeared at a dose of 300 mg), one was recovering from a cerebrovascular accident (1000 mg) and the last was mentally retarded and deafmute (800 mg). With 7 patients the enumerated symptoms disappeared within 1–4 days when treatment was stopped, while in four they did not, thus calling into question any causal inference. The daily doses at which ectodermal side effects appeared ranged from 800 to 1800 mg with a median dose of 1100 mg whereas the neurological symptoms were recorded at lower doses between 300 and 1000 mg (median 550 mg).

Laboratory abnormalities

The hemogram of all patients showed no aberrations which had not either been transient or already present at onset, signalling no bone marrow depression. Thus all of the patients with a leucocytosis or a thrombocytosis maintained them throughout the treatment.

*On a 3–4 weekly basis.

Table 1. *Clinical and laboratory data of* 11 *patients receiving VAAE*

No.	Diagnosis	Prior Rx	Age Yr/Sex	Months since diagnosis	Duration of Rx with VAAE days	Daily dose VAAE g	Total dose VAAE (g)	Clinical side effects	Laboratory abnormalities	Tumor response
1	Inoperable bronchogenic carcinoma	None	72/M	3	276	0·05–1·6	133·6	Pruritic erythematous rash at 1·6 g/day	Transient SGPT elevation up to 56 i.u.	No change
2	Metastatic testicular embryonal carcinoma	Orchidectomy radiotherapy combination chemotherapy	26/M	14	30	0·2–0·8	8·3	Perlèche, stomatitis at 0·7 g/day	None	Progression
3	Metastatic melanoma	Surgery radiotherapy	37/M	38	7	0·15–0·3	0·9	Severe headache at 0·3 g/day	None	Progression
4	Metastatic melanoma	Surgery chemotherapy	57/M	15	22	0·1–0·3	4·1	Severe headache at 0·3 g/day	Transient thrombocytosis	No change
5	Generalized arteriosclerosis	None	78/F	?	58	0·3–1·2	48·4	Stomatitis perlèche at 1 g/day	Transient BUN elevation to 59 mg %	—
6	Cerebrovascular accident gastric carcinoma	None	62/M	4	19	0·2–1·0	13·4	Pruritic erythematous rash, headache, dizziness at 1·0 g/d	Transient SGPT elevation to 42 i.u.	No change
7	Advanced mesothelioma	Combination chemotherapy	52/M	6	43	0·2–0·8	12·8	Severe headache, dizziness, vomiting at 0·8 g/day	Transient SGPT elevation to 34 i.u.	Progression
8	Resistant plasmocytoma	Chemotherapy radiotherapy	65/M	7	22	0·2–0·8	8·6	Transient headache, further Rx refused	Transient elevation of BUN, SGPT, alkaline, phosphatase	Progression
9	Metastatic breast cancer	Surgery radiotherapy oophorectomy chemotherapy	48/F	91	55	0·2–1·4	64·0	Ulcerative stomatitis at 1·4 g/day	None	Progression
10	Metastatic melanoma	Surgery radiotherapy	55/F	56	42	0·2–1·4	49·2	Perlèche, stomatitis, headache at 1·4 g/day	Leucocytosis, transient elevation of BUN to 48 mg% and SGPT to 41 i.u.	Progression
11	Metastatic bronchogenic carcinoma	None	67/M	20	72	0·4–2·0	96·0	None, Rx stopped due rapid progression	Transient elevation of alkaline phosphatase to 7·3 B.u.	Progression

In three patients there was a moderate rise in the BUN. In one it was transient and in the two others, who were severely debilitated and febrile, it is most probably explained by dehydration. A slight rise in serum uric acid was obtained in three of the nine patients in whom this value was recorded. In one it was transient and in another associated with an elevated BUN. In the third with cerebrovascular accident there was no apparent underlying cause.

The bilirubin was elevated in one patient with pancreas carcinoma at onset, and decreased with treatment along with the alcaline phosphatase and the SGPT although a control pancreas scan showed increased tumor related activity. In eight patients there were moderately elevated SGPT levels which were transient in two whereas they were already elevated at onset in the six others. In four of these they decreased moderately but steadily during treatment, while the two others had proven liver metastases. The alcaline phosphatase was elevated in 4 patients and subsequently decreased during treatment in two. In the other two, one with liver metastases and the other with a plasmocytoma, it increased moderately. The electrolytes remained within normal limits in all patients.

Therapeutic effects

Although it was not primarily the purpose of this study to assess the antitumor effect of VAAE, the presence of measurable masses or radiological densities in seven of the cases under study allowed objective data on tumor changes to be collected. In none of these was there clearcut evidence of tumor regression. Although there was some subjective improvement in two, there was increase in tumor size in a control pancreas scan of one and the other with a squamous cell carcinoma of the lung showed no objective improvement in the pulmonary X-rays. Since the main emphasis in the action of compounds of the vitamin A series lies rather in the treatment of precancerous lesions, it is not astonishing that anti-tumor effect was lacking in advanced resistant disease.

DISCUSSION

The clinical experience gathered with vitamin A acid ethylamid in man confirms the impressions gained in animal experiments. It is considerably less toxic than vitamin A acid. Whereas the latter produced side effects in man already in doses ranging from 50 to 150 mg/day, the ethylamid evinces toxicity only above 300 mg/day (usually at much higher doses). Table 1 shows that the toxicity is dependant on the daily dose and not on the total ingested amount. It is noteworthy that central nervous manifestations occurred only in patients with known organic brain disease. If these patients are excluded from the study the only untoward reactions concern skin and mouth, are for the most part tolerable and responsive to symptomatic measures and decrease of drug doses.

Predictably there is no hematotoxicity. The blood chemistry aberrations are best explained by the underlying disease (metastases, cachexia, etc.). It remains to be explained why in four patients for no apparent reason the transaminases and in two the alcaline phosphatase decreased during treatment with VAAE.

On the basis of the results presented here, vitamin A acid ethylamid can be considered a product of low toxicity. On the basis of our limited experience the proposed starting dose of VAAE for consecutive clinical trials should be 400–600 mg per day with individual increase or decrease of drug dose according to toxicity. VAAE should be used with extreme caution in doses $>$ 300 mg/day, however, in patients with organic brain disease and probably not at all in patients with cerebral metastases for its tendency to increase intracranial pressure. Barring these, the side effects most important are angular cheilitis and pruritic rashes, both amenable to symptomatic treatment or reversible with lowering the doses of VAAE at maximally tolerated doses. The effect of Vitamin A acid ethylamid taken systemically in patients with vitamin A acid responsive precancerous lesions remains to be tested clinically. VAAE might be of value as well in adjuvant therapy when taken together with alkylating agents [8].

REFERENCES

1. W. Bollag, Effects of vitamin A acid on transplantable and chemically induced tumors. *Cancer Chemother. Rep.* **55,** 53, 1971.
2. W. Bollag and F. Ott, Therapy of actinic keratoses and basal cell carcinomas with local application of vitamin A acid. *Cancer Chemother. Rep.* **55,** 59, 1971.
3. A. Schumacher and G. Stüttgen, Vitamin-A-Säure bei Hyperkeratosen, epithelialen Tumoren und Akne. *Dtsch. med. Wschr.* **96,** 1547, 1971.

4. H. J. Ryssel, K. W. Brunner and W. Bollag, Die perorale Anwendung von Vitamin-A-Säure bei Leukoplakien, Hyperkeratosen und Plattenepithelkarzinomen: Ergebnisse und Verträglichkeit. *Schweiz. med. Wschr.* **101,** 1027, 1971.
5. J.-P. Evard and W. Bollag, Konservative Behandlung der rezidivierenden Harnblasenpapillomatose mit Vitamin-A-Säure. Vorläufige Mitteilung, *Schweiz. med. Wschr.* **102,** 1880, 1972.
6. E. Brandes and D. Brandes, Studies of L-1210 leukemia, IV. Ultrastructural findings after *in vitro* treatment with cyclophosphamide and vitamin A. *Exp. molec. Path.* **7,** 156, 1967.
7. E. Anton and D. Brandes, Studies of L-1210 leukemia, IV. Ultrastructural findings after *in vitro* treatment with cyclophosphamide and vitamin A. *Exp. molec. Path.* **7,** 156, 1967.
8. M. H. Cohen and P. P. Carbone, Enhancement of antitumor effect of alkylating agents by vitamin A. *Proc. Ass. Cancer. Res.* **10,** 14, 1969.
9. W. Bollag (Unpublished data).

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Pituitary Prolactin Levels in Canine Mammary Cancer

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Abstract—*Canine pituitary prolactin concentration was estimated densitometrically after isoelectric focusing in polyacrylamide gel columns and staining by Coomassie brilliant blue. In bitches with mammary carcinoma, prolactin levels were significantly higher than in normal animals of comparable endocrine state. This finding indicated that prolactin imbalance may be involved in canine mammary neoplasia.*

INTRODUCTION

PROLACTIN is of prime importance in the genesis of mammary neoplasia in rodents [1, 2]. It is unknown, however, whether, or not, the hormone is similarly implicated in other species. There is some evidence in man that serum prolactin levels may be elevated in patients with breast cancer [3, 4], but much remains to be investigated, particularly the problem regarding the role of prolactin in the early stages of tumour induction.

In Leeds, the bitch is the subject of study of the association between prolactin and mammary neoplasia and the following report describes an investigation into the prolactin content of the adenohypophysis of bitches bearing mammary tumours.

MATERIALS AND METHODS

Animals

A series of 19 bitches, mostly mongrels, bearing spontaneous mammary tumours was used. None of these animals had received hormonal therapy. Post-mortem and histopathological examination were performed on all cases.

A series of 39 normal, sexually mature, non-pregnant bitches, again mostly mongrels, in various endocrine states, used in a previous study [5], served as a control group in this investigation.

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The reproductive state of all bitches (tumorous and control) was ascertained by external inspection combined with gross and histological examination of the reproductive organs. The dogs were destroyed by intravenous injection of sodium pentobarbitone. All control bitches and over half the tumorous cases were killed between 11.00–12.00 hours and the remainder at various times throughout the day.

Pituitaries

The adenohypophyses were removed within 30–60 min after death and either analysed for prolactin concentration immediately or stored at −20°C for intervals not exceeding 2 months (stainable canine pituitary prolactin has been shown to be durable for at least 3 months [6]). Prolactin estimations were performed by isoelectric focusing in polyacrylamide gel columns containing Ampholine carrier ampholytes of pH range 5–7 (LKB-Produkter). After completion of electrofocusing, gels were fixed in 12% trichloroacetic acid, stained with Coomassie brilliant blue and scanned densitometrically in a Joyce–Loebl Chromoscan MK II. The details of the method have been described in an earlier publication [6]. A quantity of extract equivalent to 1 mg adenohypophysis per gel was sufficient for clear detection and densitometric measurement of the prolactin band and for each gland the mean optical density of the prolactin band was calculated from the scans of eight gels and expressed as equivalent μg of ovine prolactin standard (regression analysis

had shown that ovine prolactin displayed the same dye-binding properties as canine prolactin in adenohypophysial extracts).

Statistical analysis

Previous studies on normal bitches [5] had shown that pituitary prolactin concentration varied with the endocrine state and, accordingly, the latter was taken into account when assessing levels in the tumorous bitches.

Low pituitary prolactin concentrations occurred in dioestrous females while significantly higher concentrations were found in oestrous, metoestrous (luteal) and ovariectomized animals. Significant differences did not occur amongst the oestrous, luteal or spayed categories. Accordingly, in the present study, the results for tumorous animals in oestrous, luteal and spayed conditions were pooled and compared with the mean level for normal animals in these conditions. Prolactin levels in tumorous animals in dioestrous were compared with the dioestrous level in normal animals. Tests for differences in prolactin levels were carried out by Student's *t*-test [7] using an Olivetti Programma 101 desk-top computer. In assessing estimations obtained from individual pituitaries, prolactin concentration was considered to be elevated when it was above the 95% confidence limit of the upper end of the normal range and to be depressed when it was below the 95% confidence limit of the lower end of the normal range for a given endocrine state.

RESULTS

The distribution of the cases according to tumour type, stage of disease and pituitary prolactin level is shown in Table 1.

Statistical analysis showed that, as a group, bitches with mammary tumours had significantly higher pituitary prolactin concentration than normal females of comparable endocrine state (Fig. 1). This difference was somewhat more significant among dioestrous bitches ($0{\cdot}02 > P > 0{\cdot}01$) than among oestrous–luteal–spayed animals ($0{\cdot}025 > P > 0{\cdot}02$).

Elevated prolactin levels were confined to those animals with carcinomas (Table 1).

Table 1. Description of mammary tumour cases in which pituitary prolactin concentration was measured

Number of animals	Diagnosis	Pituitary prolactin level
3	Localized infiltrating carcinoma	2N; 1E
12	Recurrent and/or metastatic carcinoma	3N; 9E
1	Recurrent, metastatic malignant mixed tumour	N
1	Recurrent, metastatic osteosarcoma	N
1	Recurrent, metastatic fibrosarcoma	N
1	Multiple pleomorphic adenomata	D

Abbreviations used: N = Normal, E = Elevated, D = Depressed.

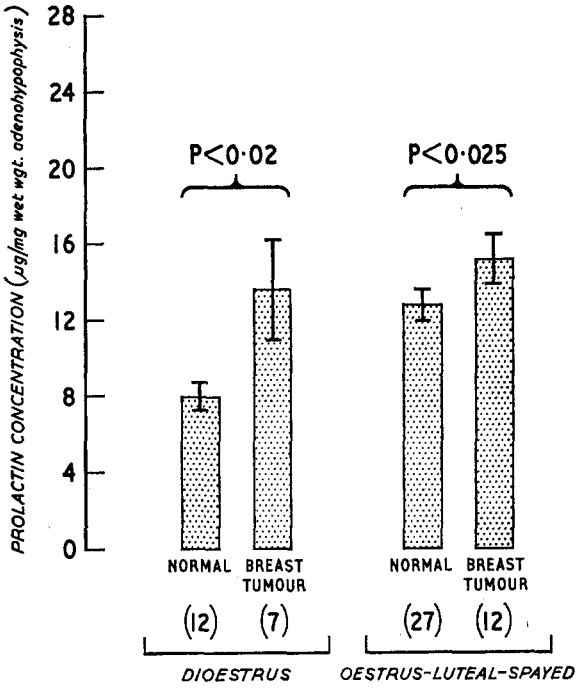


Fig. 1. Mean pituitary prolactin concentration in normal dogs and dogs with mammary tumours. The vertical bars represent S.E.M. and the numerals in brackets indicate the number of dogs examined.

DISCUSSION

The dog, a species which closely shares man's environment, suffers from spontaneous malignant mammary neoplasms at least as frequently as man [8] and, furthermore, canine and human breast cancer are remarkably similar with regard to age of onset, age specific incidence and, in many cases, histological appearance and clinical course [8, 9]. In the dog, as in the woman, there is circumstantial evidence that endocrine factors influence the incidence and course of mammary tumours

although the specific hormones involved have not been identified as yet in either species.

In the bitch a protective effect is conveyed by ovariectomy early in life [10] and also, possibly by frequent pregnancies [10, 11]. Oestrogens have been incriminated [12, 13, 14] but experimental support for this hypothesis has not been forthcoming [15, 16, 17].

Involvement of the pituitary gland is suggested by reports of enlarged adenohypophysis [18] and increased numbers of acidophils [19] in bitches bearing mammary tumours. Using differential pituitary staining, Barboni and Romanelli [20] found marked hyperplasia and hypertrophy of the prolactin cells in bitches with fibrocystic mastopathy with and without concomitant mammary cancer, as compared to normal bitches. It is not too surprising, therefore, that the above investigation has shown that bitches with mammary tumours had significantly higher pituitary prolactin concentration than normal females, although the elevation was actually confined to animals with carcinomas. Although diurnal secretory cycles for prolactin have been described in the rat [21] and the human [22], in the present study the time of death did not appear to influence the levels observed.

The mean age of the tumorous bitches was 11·8 years (±S.D. 2·7 years) with no significant age difference between oestrous–luteal–spayed bitches and dioestrous bitches ($P>0{\cdot}50$). Since precise information was not available for all control bitches, the age distribution of this series could not be presented. However, the following evidence indicated that age did not influence pituitary prolactin levels in the present study: (1) Amongst the mammary tumour bitches, there was no significant correlation between increasing age and elevation of pituitary prolactin, i.e. for oestrous–luteal–spayed bitches, $r^* = 0{\cdot}07$, $P>0{\cdot}40$ and for dioestrous bitches, $r = 0{\cdot}22$, $P>0{\cdot}35$. (2) When the control animals were classed into the broad age groups of young, intermediate and old, analysis of variance did not disclose any significant differences attributable to age. For these reasons, it was felt that the control and tumorous groups were comparable despite possible differences in age-distribution.

All animals, tumorous and normal, were subject to the same degree of acute stress with regard to handling and destruction. However the mammary tumour animals had suffered the additional stress of a serious illness. In this regard it is recognized that pituitary prolactin should be examined in dogs with serious illnesses other than mammary malignancy, although such cases were not available during the course of the present investigation.

The findings of this study suggest that prolactin may be of importance in mammary neoplasia in the dog. However, information regarding its mode of action awaits elucidation and, towards this end, methods are being developed for the measurement of serum prolactin. This, combined with assessment of pituitary content, would enable further investigation into the role of prolactin in mammary tumorigenesis in the bitch.

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*r = product moment correlation coefficient [23].

REFERENCES

1. O. H. Pearson, O. Llerena, L. Llerena, A. Molina and T. Butler, Prolactin-dependent rat mammary cancer: a model for man? *Trans. Ass. Am. Physns.* **LXXXII,** 225 (1969).
2. L. M. Boot, Prolactin and mammary gland carcinogenesis. The problem of human prolactin. *Int. J. Cancer* **5,** 167 (1970).
3. P. Berle and K. D. Voigt, Plasma-Prolaktinkonzentrationen beim mammakarzinom der frau. *Acta endocr. Copenh. suppl.* **159,** 38 (1972).
4. R. M. L. Murray, G. Mozaffarian and O. H. Pearson, Prolactin levels with L-Dopa treatment in metastatic breast carcinoma, in *Prolactin and Carcinogenesis* (Edited by A. R. Boyns and K. Griffiths), p. 158. Alpha Omega Alpha, Cardiff (1972).
5. P. G. Saluja, J. M. Hamilton and M. Gronow, Prolactin content of canine pituitary gland. *J. Endocr.* **57,** 495 (1973).
6. P. G. Saluja, M. Gronow and J. M. Hamilton, Measurement of canine pituitary prolactin. *J. Endocr.* **56,** 245 (1973).
7. J. G. Peatman, *Introduction to Applied Statistics*, p. 209. Harper & Row, Evanston and London.

8. R. Schneider, Comparison of age, sex and incidence rates in human and canine breast cancer. *Cancer (Philad.)* **26,** 419 (1970).
9. W. Misdorp, *Malignant mammary tumours in the dog and the cat compared with the same in the woman.* Doctoral thesis, Veterinary Faculty, University of Utrecht (1964).
10. R. Schneider, C. R. Dorn and D. O. N. Taylor, Factors influencing canine mammary cancer development and postsurgical survival. *J. nat. Cancer Inst.* **43,** 1249 (1969).
11. A. C. Anderson, Parameters of mammary gland tumors in aging beagles. *J. Amer. vet. med. Assoc.* **147,** 1653 (1965).
12. J. de Vitta, Mammary adenofibroma of the female dog. *N. Am. Vet.* **20,** 53 (1939).
13. G. Dozza and G. Coluzzi, Biological assay of urinary oestrogens in bitches with tumours of the mammary gland. *Atti Soc. ital. Sci. vet.* **17,** 354 (1964).
14. G. Dozza, M. L. Calandra and A. S. Li, Measurement by gas chromatography of urinary oestrogens in bitches with mammary dysplasia of neoplasia. *Nuova Vet.* **47,** 71 (1971).
15. R. M. Mulligan, Some effects of chronic doses of stilbestrol in female dogs. *Exp. Med. Surg.* **5,** 196 (1947).
16. A. Jabara, Canine mixed tumours. *Aust. vet. J.* **36,** 212 (1960).
17. A. Jabara, Canine mammary carcinomata. *Aust. vet. J.* **36,** 389 (1960).
18. C. Huggins and P. V. Moulder, Studies on the mammary tumors of dogs. I Lactation and the influence of ovariectomy and suprarenalectomy thereon. *J. exp. Med.* **80,** 441. (1944).
19. R. M. Mulligan, In *Neoplasms of the Dog.* Williams & Wilkins, Baltimore (1949).
20. E. Barboni and V. Romanelli, Remarks and considerations about fibrocystic mastopathy and about "cancer ex fibrocystic mastopathy" of the dog. In *International Symposium on Mammary Cancer* (Edited by L. Severi), p. 547. Division of Cancer Research, Perugia (1958).
21. J. D. Dunn, A. Arimura and L. E. Scheving, Effect of stress on circadian periodicity in serum LH and prolactin concentration. *Endocrinology* **90,** 29 (1972).
22. J. Nokin, M. Vekemans, M. L'Hermite and C. Robyn, Circadian periodicity of serum prolactin concentration. *Brit. med. J.* **3,** 561 (1972).
23. M. J. Moroney. In *Facts from Figures,* p. 286, Penguin Books, Harmondsworth, Middlesex.

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Further Observations on the Effect of PHA in Conjunction with Radiotherapy and Chemotherapy on S-180 in Swiss Mice

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Abstract—*Mice bearing Sarcoma–180 tumors were treated either by radiotherapy, with Vinca alkaloids or with PHA and in combination of these three treatments. Mice treated by a single form of therapy showed no significant difference from untreated control mice, but the combination of irradiation with chemotherapy proved more effective. Furthermore, PHA in combination with the two other treatments resulted in significant reduction in tumor volume, and, in some cases, prolongation of survival time.*

INTRODUCTION

THE NON-SPECIFIC stimulation of the reticuloendothelial system has been shown to affect various tumors. The growth of the methylcholanthrene induced fibrosarcoma was retarded in mice treated with neuraminidase treated tumor cells and B.C.G. (Bacillus Calmette Guerin) [1]. *C. parvum* in combination with antitumor globulin reduced the volume of mammary carcinoma in mice [2]. Patients with acute leukemia have been treated by chemotherapy and B.C.G., and better results were obtained than in patients treated with chemotherapy alone [3]. The combination of radiotherapy, chemotherapy and bacterial toxin retarded the growth of reticulum cell sarcoma in humans [4].

Previously we have reported that the supplementation by phytohemagglutinin (PHA) of the combined treatment by radiotherapy and alkylating agents reduced significantly the tumor volume and prolonged the survival of mice implanted with Sarcoma–180. It was suggested that the sequence of treatment and the selection of chemotherapeutic drugs are of great value in achieving the best results [5]. The Vinca alkaloids are widely used in the treatment of malignancy alone, either with other drugs [6, 7], or with irradiation [8]. These drugs are known as suppressors of the immune response [9]. The present investigation was undertaken to study whether the addition of PHA to the mentioned conventional methods of treatment would improve the results.

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MATERIAL AND METHODS

Mice

Swiss Albino random bred mice aged 8–10 weeks and weighing 20–22 g were used in the experiments. The mice (raised by Mr. A. Loebenstein, Yokneam, Israel), were kept in cages (15 animals per cage), and were given food and water *ad libitum*. The implantation of the tumor was carried out as described previously [5].

Irradiation

Irradiation of the tumor area was performed with the Picker Vanguard X-ray machine. The dose was calculated in roentgens (r) per mouse

in air and was given at 126 kV 5 mA with HVL of 0·6 mm Al. The mice were anesthetized by ether and were given a single dose of 2000 r in 3·9 min. The field was round in shape and 2 cm in diameter. The output was 515 r/min. The animals were irradiated at 19 cm skin target distance. The irradiation dose given to the healthy tissues was negligible.

Drugs

The following drugs were administered intraperitoneally to the mice:

1. Velbe (Vinblastine sulfate), Eli Lilly and Company, Indianapolis, Indiana. 6 μg/mouse.
2. Oncovin (Vincristine sulfate), Eli Lilly and Company, Indianapolis, Indiana. 4 μg/mouse [9].

Phytohemagglutinin (PHA)

"Wellcome" phytohemagglutinin was used (Wellcome Research Laboratories, Beckenham, England). The freeze dried material containing 50 mg/vial was stored at 4°C, and it was diluted with 5 ml of sterile saline (0·9% Nacl) before use. One mg was injected intraperitoneally per mouse. This amount has been shown to reduce tumor volume in mice bearing ascites [10].

Statistical analysis

The statistical significance of the difference in mean tumor growth and in mean survival time between different groups was tested by the standard *t*-test. When the variances in the groups compared were significantly different Bailey's modification [11] was used.

For tumor growth comparison, the average tumor volume in the period from +25 days to +30 days was divided by the average tumor volume in the period from +8 days to +13 days to give a value that represents the tumor growth for each animal. Differences between groups were considered significant only when the *P* values of comparison were 0·05 or smaller.

Experimental procedure

Two sets of experiments (each using 225 mice divided into 15 equal groups—15 mice in each) were performed. All the mice were implanted subcutaneously with the tumor in the inguinal region on day 0, and were treated when tumors were palpable as follows:

Group 1. Served as non-treated control.

Group 2. Irradiation† on the 10th day after tumor implantation.

Group 3. Chemotherapy* on the 10th day.

Group 4. PHA on the 10th day.

Group 5. Chemotherapy* on the 10th day and irradiation† on the 14th day.

Group 6. Irradiation† on the 10th day and chemotherapy* on the 14th day.

Group 7. PHA on the 10th day and chemotherapy* on the 14th day.

Group 8. Chemotherapy* on the 10th day and PHA on the 14th day.

Group 9. PHA on the 10th day and irradiation† on the 14th day.

Group. 10 Irradiation† on the 10th day and PHA on the 14th day.

Group 11. PHA on the 10th day, chemotherapy* on the 14th day and irradiation† on the 17th day.

Group 12. PHA on the 10th day, irradiation† on the 14th day and chemotherapy* on the 17th day.

Group 13. Irradiation† on the 10th day, PHA on the 14th day and chemotherapy* on the 17th day.

Group 14. Chemotherapy* on the 10th day, irradiation† on the 14th day and PHA on the 17th day.

Group 15. PHA on the 10th day, chemotherapy* on the 14th day, irradiation† on the 20th day.

RESULTS

Statistical analysis of the survival time and tumor volume obtained by one treatment such as X-rays, chemotherapy or PHA, has not revealed any significant difference as compared to the untreated controls. The results of combining two treatments (Table 1) show that Vinblastine and X-rays, whether Vinblastine was given first or X-ray first, prolonged significantly the host's survival time and reduced the tumor volume (compared to one treatment or non-treated control). The tumor volume of the mice treated with PHA and VLB was significantly lower than those treated by X-rays or VLB alone or the control whilst the survival time did not differ. No significant difference from the

*A single dose of either Velbe or Oncovin was injected intraperitoneally to the mice.

†Irradiation was given to the tumor area only.

Table 1. The effect of combining PHA, chemotherapy and X-ray treatment on the survival time and tumor volume of Swiss mice implanted with S-180 (two treatments per individual)

Group No.	Treatment	Median survival time (days)	Percentage of survivors at the end of experiment	Survival significance longer than corresponding control	Tumor volume smaller than corresponding control	Survival significance better than single treatment	Tumor volume smaller than single treatment
1	Control (for VLB Exp)	40	42				
5	VLB(D10),X-rays(D14)	*	60	+	+	X-rays(2);† VLB(3), PHA(4)	VLB(3)
6	X-rays(D10),VLB(D14)	*	60	+	+	X-rays(2); VLB(3), PHA(4)	
7	PHA(D10),VLB(D14)	50	40		+		X-rays(2), VLB(3)
8	VLB(D10),PHA(D14)	33	20				X-rays(2)
9	PHA(D10),X-rays(D14)	47	40				
10	X-rays(D10),PHA(D14)	38	40				
1	Control (for ONCOVIN Exp.)	42	27				
5	Oncovin(D10), X-rays(D14)	44	27				
6	X-rays(D10), Oncovin(D14)	*	54	+	+		
7	PHA(D10), Oncovin(D14)	43	27				
8	Oncovin(D10) PHA(D14)	46	40				

*More than 50% of the mice survived up to the end of the experiment.
†Group No.

Table 2. The effect of combining PHA, chemotherapy and X-rays treatment on the survival time and tumor volume of Swiss mice implanted with S-180 (three treatments per individual)

Group No.	Treatment	Median survival time (days)	Percentage of survivors at the end of experiment	Survival significance longer than corresponding control	Tumor volume significance smaller than corresponding control
1	Control (VLB exp.)	37	23		
11	PHA(D10),VLB(D14),X-rays(D17)	50	46		
12	PHA(D10),X-rays(D14),VLB(D17)	*	93	+	+
13	X-rays(D10),PHA(D14),VLB(D17)	*	64	+	+
14	VLB(D10),X-rays(D14),PHA(D17)	24	27		+
15	PHA(D10),VLB(D14),X-rays(D17),PHA(D20)	56	46		+
1	Control (Oncovin exp.)	37	27		
11	PHA(D10),Oncovin(D14),X-rays(D17)	36	34		
12	PHA(D10),X-rays(D14),Oncovin(D17)	49	46		+
13	X-rays(D10),PHA(D14),Oncovin(D17)	44	34		+
14	Oncovin(D10),X-rays(D14),PHA(D17)	*	54	+	+
15	PHA(D10),Oncovin(D14),X-rays(D17), PHA(D20)	43	40		+

*More than 50% of the animals survived up to the end of the experiment.

Table 3. *The effect of combining three treatments compared to one treatment and two treatments on the survival time and tumor volume of Swiss mice implanted with S–180*

Group No.	Treatment	Median survival time (days)	Percentage of survivors at the end of experiment	*P* value	Tumor volume on the 30th day (cm^3)	*P* value
	Single treatment					
1.	Control	42	34		0·70	
2.	X-rays (D10)	45	40		1·10	
3.	VLB (D10)	41	47		0.70	
3a.	Oncovin (D10)	48	40		1·70	
4.	PHA (D10)	32	34		1·70	
	Combined treatments					
5.	VLB(D10),X-rays(D14)	*	60	0·02(1†) 0·02(2) 0·01(3)	0·22	0·001(1) 0·02(3)
6.	X-rays(D10),VLB(D14)	*	60	0·02(1)	0·58	0·02(1)
11.	PHA(D10),VLB(D14),X-rays(D17)	50	46		0·78	
12.	PHA(D10),X-rays(D14),VLB(D17)	*	93	0·01(1)	0·59	0·02(1)
13.	X-rays(D10),PHA(D14),VLB(D17)	*	60	0·02(1) 0·001(2) 0·002(3) 0·02(4)	0·20	0·001(1) 0·02(2) 0·02(3)
14.	VLB(D10),X-rays(D14),PHA(D17)	24	27		0·18	0·02(1)
15.	PHA(D10),VLB(D14),X-rays(D17), PHA(D20)	56	46	0·002(1) 0·005(2) 0·05(3) 0·02(4)	0·29	0·02(1) 0·001(3)

*More than 50% of the mice survived up to the end of the experiment.
†Group No.

Table 3(*a*). *The effect of combining three treatments compared to one treatment and two treatments on the survival time and tumor volume of Swiss mice implanted with S–180*

Group No.	Treatment	Median survival time (days)	Percentage of survivors at the end of experiment	*P* value	Tumor volume on the 30th day (cm^3)	*P* value
	Combined treatments (cont'd)					
5.	Oncovin(D10),X-rays(D14)	44	27		0·51	
6.	X-rays(D10),Oncovin(D14)	*	54	0·02(1†)	0·01	0·01(1)
7.	PHA(D10),Oncovin(D14)					0·02(1) 0·05(3)
11.	PHA(D10),Oncovin(D14),X-rays(D17)	36	34		0·12	0·02(2)
12.	PHA(D10),X-rays(D14),Oncovin(D17)	49	46		0·06	0·03(2) 0·02(7)
13.	X-rays(D10),PHA(D14),Oncovin(D17)	44	34		0·09	0·002(3) 0·002(4)
14.	Oncovin(D10),X-rays(D14),PHA(D17)	*	54	0·02(1)	0·19	0·001(1)
15.	PHA(D10),Oncovin(D14),X-rays(D17) PHA(D20)	43	40		0·05	0·02(1) 0·001(2) 0·01(7)

*More than 50% of the mice survived up to the end of the experiment.
†Group No.

control was found in the survival time and tumor volume of mice treated with X-rays and PHA in either order, VLB and PHA. The combination of VLB and PHA was significantly better than radiotherapy alone. The various combinations of two treatments such as PHA and X-rays, or Oncovin and PHA, did not improve the results obtained by a single treatment. The combination of X-ray treatment followed by Oncovin administration, however, resulted in more than 50% of the animals surviving up to the end of the experiment.

Table 4 that in most cases results obtained by three methods of therapy were significantly better than single or two ways of treatment.

All the mice, including those treated by combined therapy were in good health. No signs of host toxicity or severe body weight loss were observed during the experiment.

DISCUSSION

We have reported on the effect of PHA on ascites tumors in mice [10]. It was therefore of

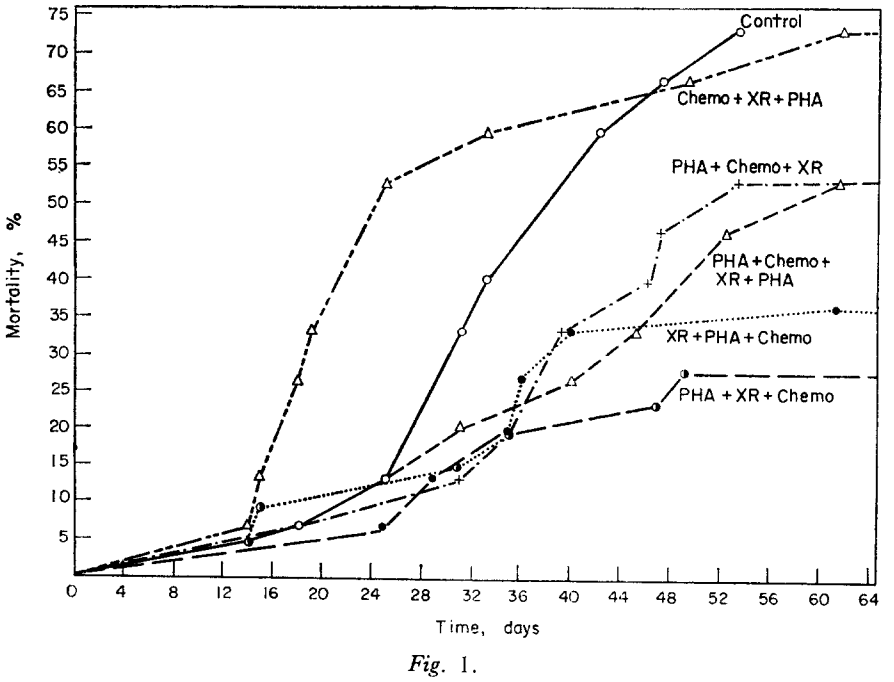


Fig. 1.

The effect of three treatments on the survival time and tumor volume are summarised in Tables 2, 3 and 3(a). In the groups were VLB served as the chemotherapeutic agent, the sequence PHA, X-rays, VLB, was the best treatment in prolongation of survival time (Fig. 1) and in reduction of the tumor growth (Fig. 2). The sequence X-rays, PHA, VLB, was the second best treatment. The two combinations VLB, X-rays, PHA, and PHA, VLB, X-rays, reduced significantly the tumor volume but had no statistically significant effect on the survival time.

In the series where Oncovin was introduced in combination with X-rays and PHA, the sequence Oncovin, X-rays, PHA, resulted in a significant prolongation of survival time (more than 50% survived up to the end of the experiment) and reduced significantly the tumor volume compared to untreated control. Two other sequences such as Oncovin given after X-rays and PHA, or Oncovin given after PHA and X-rays (Table 2), retarded significantly the tumor growth. It is evident from

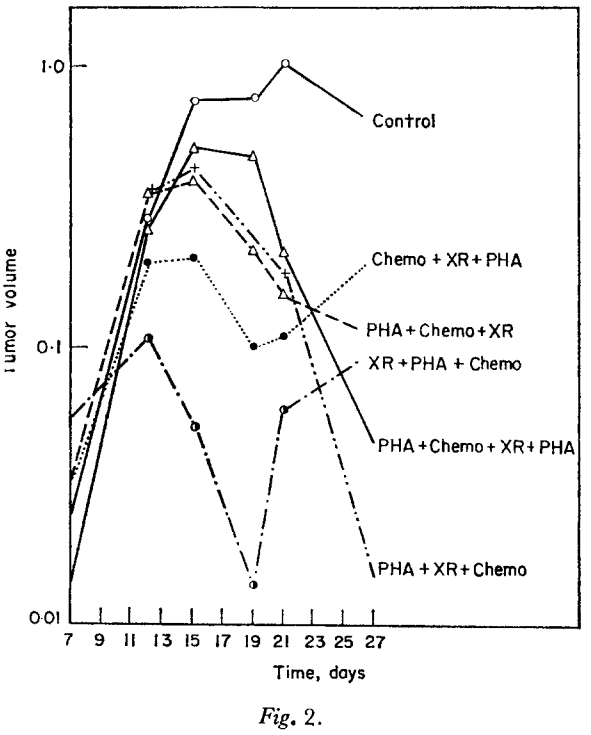


Fig. 2.

Table 4. Statistical analysis of the results obtained by three treatments

Group No.	Treatment	Survival significantly longer than	Tumor volume significantly lower than
13.	X-rays(D10),PHA(D14),VLB(D17)	VLB,X-rays,PHA(14)	X-rays(2) PHA,X-rays,VLB(12)
14.	VLB(D10),X-rays(D14),PHA(D17)		X-rays(2) PHA,X-rays,VLB(12)
15.	PHA(D10),VLB(D14),X-rays(D17)PHA(D20)	VLB,X-rays,PHA(14)	VLB(3) X-rays,VLB(6) PHA,X-rays,VLB(12)
11.	PHA(D10),Oncovin(D14),X-rays(D17)		X-rays(2)
12.	PHA(D10),X-rays(D14),Oncovin(D17)		X-rays(2) PHA,VLB(7)
13.	X-rays(D10),PHA(D14),Oncovin(D17)		PHA,VLB(7)
15.	PHA(D10),Oncovin(D14),X-rays(D17)PHA(D20)		X-rays(2) PHA,VLB(7)

interest to study whether it had the same effect on solid tumors. We have chosen the S-180 in nonbred mice as a model system.

In a previous paper [5] we have reported that mice bearing Sarcoma–180 tumors showed a significantly longer survival time and a retardation of tumor growth when treated by radiotherapy together with alkylating agent supplemented by PHA. It was therefore found that the sequence of application of the treatments is an important factor. We might assume that the suppressive activity of alkylating agents did not alter the immune stimulation by PHA. The purpose of the present investigation was to study the effect of PHA administrations to mice with irridiated tumor and given another chemotherapeutic drug.

The results show the PHA alone enhanced tumor growth. The tumor volume of the treated mice was 1·70 cm^3 compared to 0·70 cm^3 of the controls (Table 3). The same effect was recently observed by Wagener [13] in mice with fibrosarcoma. We have found that in 8 out of 10 different sequences, the consequence of combining three different methods of therapy, X-irradiation, chemotherapy and phytohemagglutinin had a significant retardation of tumor growth. In three cases a marked prolongation of survival rate was achieved. Using the different chemotherapeutic drugs, we have found that a single treatment by a single drug was not effective. Skeel and Bertino [14] have reported on prolongation of survival time of mice with S-180 after combination chemotherapy with methotrexate and bleomycin. The present results confirm other reports that the combined treatment of an effective antitumor drug with a nonspecific immune stimulator is more effective than either treatment alone. It provides another proof that the suppressive activity of the Vinca alkaloids did not interfere with the immune stimulation by PHA. Our data indicate that PHA administration 3 days after chemotherapy produced a significantly higher percentage of long term survivors. The same effect was found in a different system by Pearson *et al.* who have reported on 76% complete remission in the case of murine lymphoid leukemia treated with BCNU and 3 days later *Corynbacterium granulosum* [15].

It would be noticed that the percentage of mortality at the end of the experiment reached the value of 73–77%. These data are in accordance with other investigators who report on spontaneous regression of S-180 occurring in 26% of the random bred Swiss mice [16]. We may expect spontaneous regression of human tumors based on the theory of "immunological surveillance" [17]. Observation of occasional spontaneous regression are evident for defence mechanisms in man against neoplastic disease [18].

Many authors suggest that immunostimulators may prove to be valuable adjuncts to chemotherapy when tumor control is based on stimulation of host immunity. There is no definitive explanation for the mechanism of the immune stimulation, but factors such as antigenicity of tumors, rate of cell replication before and after drug therapy, the immune status of the host, may change the effectiveness of the stimulators. Using BCG as for immuno-

therapy in the case of rat tumors, Baldwin and Pimm [19] claimed that host's response was dependent upon the immunogenicity of the treated tumors. Their findings indicated that immunotherapy alone could be effective only to highly immunogenic tumors, whereas the same treatment caused tumor enhancement in several weak tumors including a spontaneously arising sarcoma which is similar to the above described S-180. Some authors are in favor of enhancing the tumor's specific response by irradiated tumor cells [20], while others prefer stimulating primarily non specific elements of the RES like *C. granulosum* [15] and *C. parvum*, or by cell walls of *Mycobacterium bovis* attached to oil droplets [21]. There are some indications for immune reactivity towards human spontaneous tumors [3], although the nature, the dose, the route of administration and the crucial time for starting immunostimulation are still questionable. With any effective stimulant of antitumor immune responses, one must be concerned with the possibility of immunological enhancement of tumor growth. It is therefore of special interest that in all experiments, where PHA has been given in combination with radiation and chemotherapy, enhancement did not occur. It is difficult to expect that host immune reactivity towards spontaneous tumors could be stimulated in a similar way. In our system so far, we treat animals with transplanted tumors, whereas such a problem should be investigated on primary authochtoneous tumor before any application to the human therapy.

It still remains an open question concerning the contradictory evidence on the mechanism of PHA activation in the humoral and cellular mediated immunity [23, 24]. Describing experiments on the use of S-asparaginase on a mouse lymphoma, Carter and Connors have also concluded that such a success was dependent on the host's immune response [22]. Experiments are being performed at present to clarify the cellular basis for non specific stimulation of tumor bearing animals with PHA. Studies are carried out in our laboratory to investigate similar problems in syngeneic tumor systems.

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REFERENCES

1. R. L. Simmons and A. Rios, Immunotherapy of Cancer: Immunospecific rejection of tumors in recipients of neurominidase-treated tumor cells plus BCG. *Science* **174,** 591 (1971).
2. M. F. A. Woodruff, M. P. Inchley and N. Dunbar, Further observations on the effect of *C. parvum* and anti-tumor globulin on syngeneically transplanted mouse tumors. *Brit. J. Cancer* **26,** 67 (1972).
3. G. Mathé, Immunological treatment of leukaemias. *Brit. med. J.* **IV,** 487 (1970).
4. T. R. Miller and J. T. Nicholson, End results in reticulum cell sarcoma of bone treated by bacterial toxin therapy or combined with surgery and/or radiotherapy (47 cases) or with concurrent injection *Cancer (Philad.)* **27,** 524 (1971).
5. E. Robinson and T. Mekori, Combinations of the effects of radiotherapy, alkylating agents and phytohemagglutinin on Sarcoma-180 in Swiss mice. *Europ. J. Cancer* **9,** 125 (1973).
6. A. M. Marmont and E. E. Damesio, The effects of two alkaloids derived from *Vinca rosea* on the malignant cells of Hodgkin's disease. Lymphosarcoma and acute leukaemia *in vivo*. *Blood* **29,** 1 (1967).
7. M. J. Lacher and J. R. Durant, Combined vinblastine and chlorambucil therapy of Hodgkin's disease. *Ann. int.* **62,** 648 (1965).
8. T. J. Vietti, M. P. Sullivan, M. E. Haggard, T. M. Holcomb and D. H. Berry, Vincristine sulfate and radiation therapy in metastatic Wilm's tumor. *Cancer (Philad.)* **25,** 12 (1970).
9. A. C. Aisenberg and B. Wilkes, Studies on the suppression of immune responses by the Periwinkle Alkaloids Vincristine and Vinblastine. *J. clin. Invest.* **43,** 2394 (1964).
10. E. Robinson and T. Mekori, Studies on the effect of phytohemagglutinin on ascites tumor in mice. *Israel J. med. Sci.* **7,** 83, (1971).

11. N. T. Bailey, *Statistical Methods in Biology*. English University Press, London (1959).
12. H. A. M. Attia and D. W. Weiss, Immunology of spontaneous mammary carcinoma in mice. Acquired tumor resistance and enhancement in strain "A" mice infected with mammary tumor virus. *Cancer Res.* **26,** 1487 (1966).
13. D. J. Theo Wagener, Enhanced growth of fibrosarcoma in mice treated with phytohemagglutinin. *Cancer Res.* **33,** 1295 (1973).
14. R. T. Skeel and J. R. Bertino, Combination chemotherapy of Sarcoma-180 with methotrexate and bleomycin. *Cancer Res.* **33,** 1028 (1973).
15. J. W. Pearson, G. R. Pearson, W. T. Gibson, J. C. Cherman and M. A. Chirigos, Combined chemoimmunostimulating therapy against murine leukaemia. *Cancer Res.* **32,** 904 (1972).
16. B. Shohat and B. Joshua, Host defence mechanisms in the spontaneous regression of S-180. *Europ J. Cancer* **5,** 69 (1969).
17. R. M. Burnet, Implications of immunological surveillance for cancer therapy. *Israel J. med. Sci.* **7,** 7 (1971).
18. T. C. Everson and W. H. Cole, Spontaneous Regression of Cancer. W. B. Saunders, Philadelphia (1966).
19. R. W. Baldwin and M. V. Pimm, BCG immunotherapy of pulmonary growths from intravenously transferred rat tumor cells. *Brit. J. Cancer (Philad.)* **27,** 48 (1973).
20. P. C. L. Beverley, R. M. Lowenthal and D. A. J. Tyrell, Immune responses in mice to tumor challenge after immunization with Newcastle disease virus-infected or X-irradiated tumour cells or cell fraction. *Int. J. Cancer (Philad.)* **11,** 212 (1973).
("Caution is advocated in applying the results of experiments with such animal tumour virus models to the human clinical situation".)
21. B. Zbar, J. H. Rapp and E. E. Ribi, Tumour suppression by cell walls of *Mycobacterium bovis* attached to oil droplets. *J. nat. Cancer Inst.* **48,** 831 (1972).
22. R. L. Carter, T. A. Connors, B. J. Weston and A. J. S. Davies, Treatment of a mouse lymphoma by L-asparaginase: success depends on the host's immune response. *Int. J. Cancer* **11,** 345 (1973).
23. K. Markely, S. W. Thornton and E. Smallman, On the mechanism of action of phytohemagglutinin in cellular immunity. *Proc. Soc. exp. Biol.* (N.Y.) **139,** 37 (1972).

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Selective Inhibition of the Growth of Bacteriophage Lambda by Experimental Antileukaemic Bisquaternary Salts

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Abstract—*A study was made of members of a series of pyridinium 3,3'-[(terephthaloyl bis(imino-p-phenylenecarboxylamino)] bis(1-alkyl) salts, some of which show high activity against the murine L1210 leukaemia. The inhibitory effects against* E. coli *and bacteriophage lambda were compared. The more lipophilic members of the series (alkyl = butyl or pentyl) were more effective in inhibiting bacterial growth, protein and nucleic acid synthesis. However the hydrophilic members (alkyl = methyl, ethyl or propyl), at concentrations only slightly inhibitory to the host bacterium, strongly inhibited the production of phage lambda. The di-ethyl salt inhibited phage λc_{1857} production following heat induction by 99·98% and following infection by at least 99%. The order of activity of the members of the drug series against the phage system was similar to the order of antileukaemic activity.*

INTRODUCTION

CAIN and co-workers [1] have synthesized a large number of bisquaternary ammonium heterocycles which demonstrate high activity against murine leukaemia L1210. The necessary structural features for antileukaemic activity in these agents are two cationic functions separated at least 18·5Å by a relatively rigid, coplaner aromatic skeleton, an example of which is shown in Fig. 1. The agents investigated here comprise an homologous series [2] of symmetric di-N-*n*-alkyl quaternary salts (Fig. 1. $R = CH_3$; C_2H_5; $(CH_2)_2CH_3$; $(CH_2)_3CH_3$; $(CH_2)_4CH_3$) which have been abbreviated in the text as Me, Et, Pr, Bu and Pe respectively.

The physicochemical properties of these agents play a crucial role in determining the antileukaemic activity. There is a parabolic dependence of activity against the L1210 leukaemia (growing in C_3H/DBA_2F_1 hybrid mice) on the lipophilic–hydrophilic balance as measured by solvent partitioning; the order of effectiveness is Et>Me>Pr>Bu>Pe [2]. The Et derivative is a powerful antileukaemic agent in mice.

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In attempting to account for the mode of action of these agents, and for the dependence of activity on physicochemical properties, the action of the series of drugs on a number of *in vitro* and *in vivo* systems is being investigated. One of the most interesting results with a compound closely related to the above series has been obtained with the Rous sarcoma virus (H. Diggelman, personal communication). The compound has been shown to resemble rifampicin [3] in preventing virus induced transformation of culture chick embryo fibroblasts, at concentrations well below those inhibiting growth of cells. Selective anti-viral effects would be of great interest in explaining the action of an anti-tumour drug.

The present studies have compared the effects of the drug series in Fig. 1 on *Escherichia coli* with those on one of its viruses, bacteriophage lambda. This bacteriophage has often been compared to tumour viruses in being able to exist in a prophage stage. Our results show that the drug series exert selective toxicity towards the development of the bacteriophage following induction or infection of bacteria. Furthermore, a parabolic dependence of anti-bacteriophage activity on the lipophilic–hydrophilic balance of the agents has been observed.

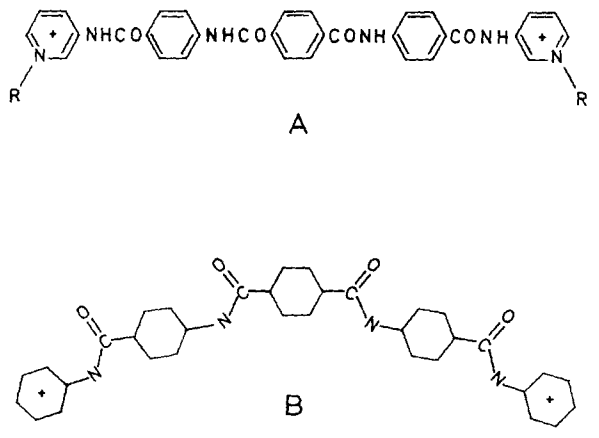


Fig. 1. Structure of the members of the drug series studied. A—Chemical structure of pyridinium 3,3′-(terephthaloyl bis-(imino-p-phenylenecarboxylamino)) bis (1-alkyl-p-tolune sulphonate). In compounds designated in the text as Me, Et, Pr, and Bu respectively, alkyl stands for methyl, ethyl, propyl and butyl respectively. The pentyl derivative (Pe) was prepared as the dibromide rather than the di-p-toluene sulphonate. B—Suggested planar conformation of the drugs [1].

MATERIAL AND METHODS

Agents

Synthetic methods for the preparation of Me, Et, Pr, and Bu have been described [2]. Samples were examined by paper chromatography before use to ensure that no decomposition had occurred. The Pe homolog was synthesized as the dibromide by the same method and crystallised from aqueous ethanol-sodium bromide as colourless needles, mp 321°C (decomp.). Chemical analysis confirmed the expected formula of $C_{42}H_{42}N_6O_4Br_2$. When administered to mice bearing the L1210 leukaemia (60 mg/kg per day, once daily for five days intraperitoneally) it did not extend the life of leukaemic mice. Higher doses were toxic.

The compounds Me–Pe were dissolved in water by warming at a concentration of 0·2 mg/ml, giving concentrations of 0·26 mM, 0·25 mM, 0·26 mM, 0·24 mM and 0·23 mM for Me, Et, Pr, Bu and Pe respectively. The molar extinction coefficient of these compounds at 305 mμ was 4×10^4.

Bacterial strains

PB64 xyl$^-$, thi$^-$, thy A$^-$, c_{1857} endonuclease I$^-$.

PB151 F$^-$, *thi-19, lac-15, leu-21, thr-13,* str-r, resistant to T5.

PB408 F$^-$, *trp-21, tsm-8, lac-26, str-95,* requires thr, leu and ile.

Bacterial cultures were kept as suspensions in 56/2 medium at 4°C, and were sub-cultured every two months.

Culture media

One litre of L broth contained Difco Bacto-tryptone (10 g), Difco Yeast Extract (5 g) and NaCl (10 g) and sufficient NaOH to adjust the pH to 7·0.

Casamino acids medium contained per litre NH_4Cl (2 g), NaCl (3 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), $MgCl_2 \cdot 6H_2O$ (83 mg), Na_2SO_4 (115 mg), Casamino acids (10 g), thymidine (10 mg), thiamine (1 mg) and glucose (added separately) 1%.

Medium 56/2 (pH 7·0–7·2) contained per litre KH_2PO_4 (2·68 g), $Na_2HPO_4 \cdot 7H_2O$ (8·2 g), $(NH_4)_2SO_4$ (1 g), $MgSO_4 \cdot 7H_2O$ (0·1 g), $Ca(NO_3)_2$ (5 mg), $FeSO_4 \cdot 7H_2O$ (0·14 mg).

L–O agar consisted of L broth containing 2·2% agar.

Estimation of culture growth

Bacterial growth was followed by measuring the absorbance at 650 mμ. Viable cells were measured by plating appropriate dilutions of the culture (in 56/2 medium) on L–O agar. 0·5 A_{650} units per ml corresponded to about 3×10^8 cells/ml in Casamino acids medium, and about 10^9 cells/ml in L broth.

RESULTS

Effects of the drug series on growth of Escherichia coli

E. coli PB64, containing the lysogenic bacteriophage c_{1857} was grown in L broth at 30°C to an absorbance at 650 mμ of 0·14. The compounds Me–Pe were added at various concentrations and incubation was continued to ascertain the effect of the drugs on increase in absorbance (Fig. 2). At all concentrations of Me, Et and Pr studied (1–10 μg/ml) and at lower concentrations of Bu and Pe (1–2 μg/ml) growth, as measured by increase of absorbance, was decreased. The same effect was seen when PB64 or PB408 were grown in casamino acids medium under the same conditions. The Et homolog at a concentration of 10 μg/ml decreased growth by about 50% in all cases. It also had the same effect on the growth of PB151 (in L broth) both at 30°C and 42°C, even though the doubling times of PB151 at 30°C and 42°C were 50 min and 35 min respectively.

A cytolytic effect, observed as a rapid decrease in absorbance and cell viability, occurred with the Bu and Pe derivatives at 5 μg/ml and 10 μg/ml. This was seen with PB64 (Fig. 3B and 3C) and also with PB408 (not shown) in both L broth and casamino acids medium.

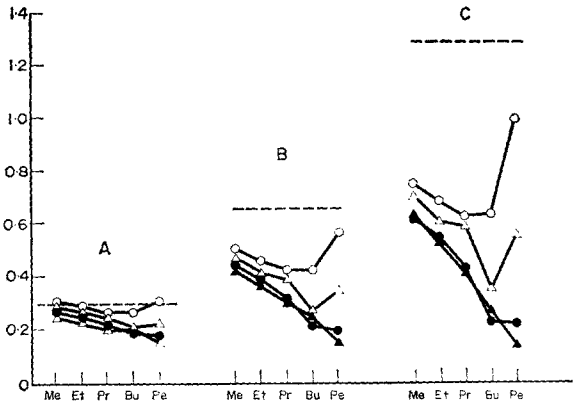


Fig. 2. The effect of the drug series on the growth of E. coli *PB64 in L broth.*

A culture of E. coli *PB64 was grown at* 30° *to* 7×10^7 *cells/ml. The Me, Et, Pr, Bu and Pe derivatives were added at concentrations of* 1 *μg/ml* (○—○, 2 *μg/ml* (△—△), 5 *μg/ml* (●—●) *and* 10 *μg/ml* (▲—▲) *and the absorbance at* 650 *mμ* (*initially* 0·1) *was measured* (*A*) 1 *hr after drug addition,* (*B*) 2 *hr after drug addition and* (*C*) 3 *hr after drug addition. Dashed lines show absorbances of control cultures.*

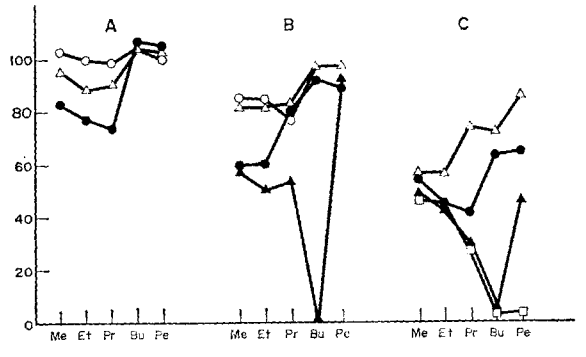


Fig. 3. The effect of the drug series (Me–Pe) on the optical density of E. coli *at stationary phase.*

Cultures of PB408 and PB64 were grown from an absorbance at 650 *mμ of* 0·1 *to stationary phase. Absorbances at stationary phase are expressed as percentage of the absorbance reached by the control culture.* (*A*) E. coli *PB408 in casamino acid medium.* (*B*) *PB64 in* c *casamino acid medium.* (*C*) *PB64 in L broth. Percentages expressed after growing in* 0·5 *μg/ml* (○—○), 1 *μg/ml* (△—△), 2 *μg/ml* (●—●), 5 *μg/ml* (▲—▲), *and* 10 *μg/ml of the drugs* (□—□). *Absorbances* (650 *mμ*) *of control cultures at stationary phase were* (*A*) 1·36, (*B*) 3·88 *and* (*C*) 3·00.

At a concentration of 2 μg/ml, Me, Et and Pr caused all three strains of *E. coli* to reach stationary phase at a lower absorbance than control cultures. The Bu and Pe compounds had little effect (Fig. 3).

Effects of the drug series on synthesis of nucleic acids proteins

To investigate the inhibition of DNA synthesis, it was preferable to use a method which gave a linear incorporation of ^{3}H-thymidine over at least one generation time. The strain PB408, which lacks thymidine phosphorylase, does not degrade thymidine, and *E. coli* mutants lacking this enzyme, unlike wild type *E. coli*, incorporate ^{3}H-thymidine linearly [4]. PB408 incorporated ^{3}H-thymidine linearly for at least two hours. Of the drug series, Bu inhibited ^{3}H-thymidine incorporation most efficiently (Pe was not tested) although all members of the series inhibited incorporation at sufficiently high concentrations (Fig. 4).

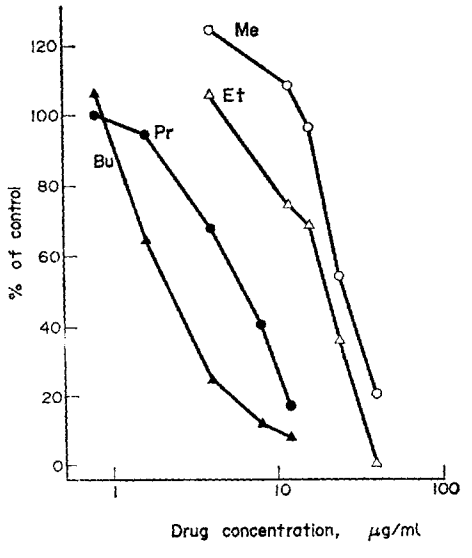


Fig. 4. Effect of the drug series on ^{3}*H-thymidine incorporation in* E. coli *PB408.*

Cultures were grown in casamino acids medium to a density of $6{\cdot}5 \times 10^7$ *cells/ml.* ^{3}*H-thymidine* (*specific activity* 20 *Ci/mmole*) *was added at a concentration of* 0·8 *μCi/ml, and* 1 *ml portions were added to tubes containing* 250 *μl of water plus the indicated drug concentrations. After incubation at* 30° *for* 1 *hr each sample was treated with cold* 10% *trichloroacetic acid, kept at* 0° *for* 30 *min, and filtered through a Sartorius membrane filter. Filters were washed with* N *HCl* (3 × 10 *ml*), *dried and the radioactivity measured in a liquid scintillation spectrometer. Radioactivity was corrected for zero time by filtering corresponding samples which had not been incubated at* 30°. *Results are expressed as a percentage of a control sample (no drug) which incorporated* 10,700 *c/m in* 1 *hr. Inhibition curves are shown for the Me derivative* (○—○), *Et derivative* (△—△), *Pr derivative* (●—●) *and Bu derivative* (▲—▲).

The effects of the drug series on the incorporation of ^{14}C-uridine and ^{14}C-valine, used as a measure of RNA and protein synthesis, were also measured, and are summarized in Table 1. They were comparable to the effects on DNA synthesis. In a separate experiment it was found that inhibition of growth by the Et homolog (as measured by absorbance) paralleled the inhibition of DNA synthesis.

Table 1. *Comparison of the effects of the drug series Me, Et, Pr, Bu and Pe on nucleic acid and protein synthesis in* E. coli *PB*408, *growing in casamino acids medium for* 30 *min at* 30°C

	Drug tested				
	Me	Et	Pr	Bu	Pe
DNA*	15	15	7	3·5	—
RNA†	10	10	7	1·5	1·0
Protein‡	10	10	9	1·5	1·5

*Incorporation of ^{3}H-thymidine (20 Ci/mmole, 0·8 μCi/ml) into material insoluble in cold 5% trichloroacetic acid. Conditions are otherwise similar to those described in Fig. 4.

†Incorporation of ^{14}C-uridine (60 mCi/mmole, 0·25 μCi/ml) into material insoluble in cold 5% trichloroacetic acid.

‡Incorporation of ^{14}C-leucine (60 mCi/mmole, 0·25 μCi/ml) into material insoluble in hot 5% trichloroacetic acid.

The table shows the concentrations (μg/ml) required for 50% inhibition of incorporation of the radioactive precursor.

Effects of the drug series on the induction of phage lambda

Induction of the lysogenic form of phage λ is normally carried out by treating bacteria with ultraviolet light or drugs such as mitomycin C. Because of possible complications if such procedures were used in combination with treatment with the series of drugs under study, a heat inducible phage mutant, λc_{1857}, was used for all experiments. It existed in lysogenic form in PB64, and was induced by heating a culture from 30°C to 42°C for 15 min to denature the phage repressor, and then incubating for a further 105 min at 37° to allow phage production and lysis of the host.

E. coli PB64 was grown at 30°C in L broth to a cell density of 10^9 cells/ml (an absorbance at 650 mμ of 0·5) and treated with one of the compounds Me–Pe. After 5 min the cultures were induced and the phage yield was measured. The results (Fig. 5) indicate that all the compounds inhibited phage production, but that Me, Et and Pr were the most potent, decreasing the phage yield by 99–99·98% at 1–2 μg/ml. Further incubation of induced cultures in the presence of Et homolog for up to 6 hr did not increase the yield of phage.

In a second experiment, PB64 was grown at 30°C in casamino acids medium to 3×10^8 cells/ml and diluted 10-fold into medium at 42°C containing the Et homolog (1 μg/ml). The phage yield was decreased by 99·5% (the control cultures produced 50 phage per bacterium).

The effect of the timing of addition of drugs was investigated. Addition of the Et derivative to PB64 in L broth more than one minute after induction was much less effective (Fig. 6). On the other hand, addition up to 1 hr before induction did not increase the extent of inhibition.

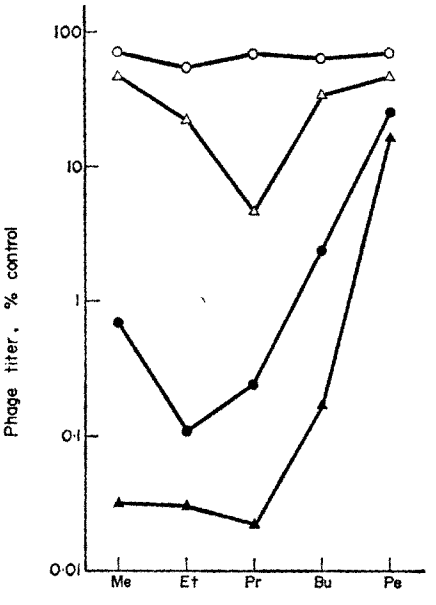


Fig. 5. *Effect of the drug series on the induction of bacteriophage lambda.*

E. coli *PB*64, *containing the heat-inducible phage* λc_{1857}, *was grown at* 30° *in flasks containing* 4 *ml of L broth. At a cell density of* 10^9 *cells/ml the derivatives (Me, Et, Pr, Bu, and Pe) were added at concentrations of* 0·25 μg/ml (○—○), 0·5 μg/ml, (△—△), 1 μg/ml (●—●) *and* 2 μg/ml (▲—▲). *Five min later the cultures were heated to* 42°C *for* 15 *min and were further incubated at* 37° *for* 105 *minutes. The cultures were shaken with a few drops of chloroform and the phage yield was determined by plating on agar using* E. coli *PB*151 *as indicator strain. The control culture yielded* $5{\cdot}6 \times 10^{10}$ *plaque-forming units/ml. The titres of the drug-treated cultures are expressed as percentages of the control.*

Absorbance measurements of induced cultures of PB64 in L broth showed that the presence of the Et homolog made no difference up to the time at which lysis of the control culture occurred. At this time a slight decrease (8%) in the absorbance of the drug treated culture occurred, and thereafter the absorbance increased slowly (25% over the next 90 min).

By determining the number of bacteria surviving after induction in the presence and absence of the Et compound it was possible to see if the prophage had maintained its lysogenic state. It was found that induction in the

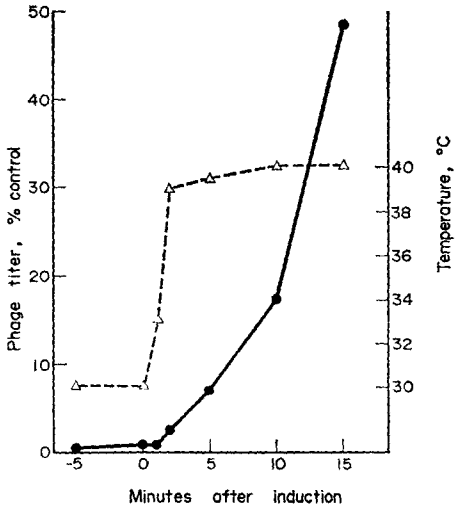


Fig. 6. Effect of variation of the time of addition of the Et derivative on the yield phage.

The drug was added to 4 ml portions of PB64 in L broth at the indicated times. Cultures at a density of 10^9 cells/ml were shifted to a water bath at 42° and the culture temperature was measured (– – – –). After 15 min, the cultures were incubated at 37° for 10 min. The phage titres of the drug-treated cultures (●—●) are expressed as percentages of the control culture titre ($3{\cdot}6 \times 10^{10}$ plaque-forming units/ml).

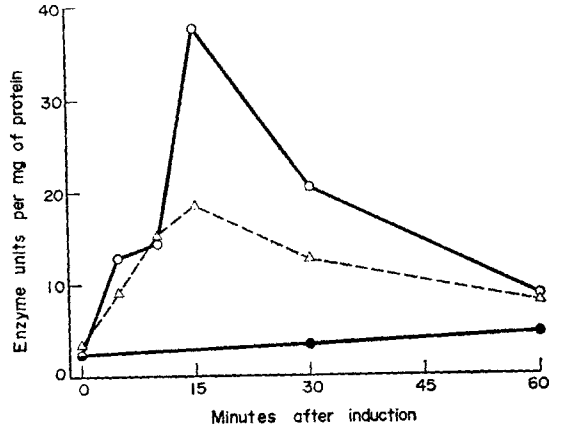


Fig. 7. Induction of exonuclease activity following phage induction in the presence and absence of the Et derivative.

E. coli *PB64 was grown to a density of* 2×10^9 *cells/ml and the Et derivative was added to one culture at a concentration of 2 μg/ml. After 5 min the cultures were heated to 42°C and samples (50 ml) were taken at intervals. Samples were cooled to 0°, centrifuged (5000 g, 10 min., 0°) and resuspended in 0·5 M glycine buffer, pH 9·6 (5 ml). The samples were then assayed for exonuclease activity by the procedure of Radding [7]. The bacterial cells were disrupted by passage through a needle valve at* 2×10^4 *kilopascals, and the cell extract was centrifuged (30,000 g, 15 min, 0°) before assay. The specific activity of the exonuclease was measured at various times in the control induced culture (○—○), the drug-treated induced culture (△– – –△), and the control uninduced culture grown at 30° (●—●).*

presence of drug killed the same proportion of bacteria (99·96%) as induction of control cultures. Attempts were made to "rescue" the bacteria by returning the cultures to 30°C after 15 min at 42°C, or by centrifuging and resuspending the cells in fresh L broth containing no drug after 15 min at 42°C, but these attempts were unsuccessful. The latter treatment also did not increase the phage yield.

One of the enzymes made soon after induction of phage λ is an exonuclease. To determine how soon drug effects were evident on bacteriophage function, this enzyme was assayed in cultures of PB64 in L broth. The results (Fig. 7) show that the maximum exonuclease activity, obtained 15 min after induction, was 50% lower in the drug treated culture.

The rate of DNA synthesis rises following induction [5]. To examine the effect of the Et homolog on this process, ^{3}H-thymidine incorporation during one minute pulses was measured at different times after induction (Fig. 8).

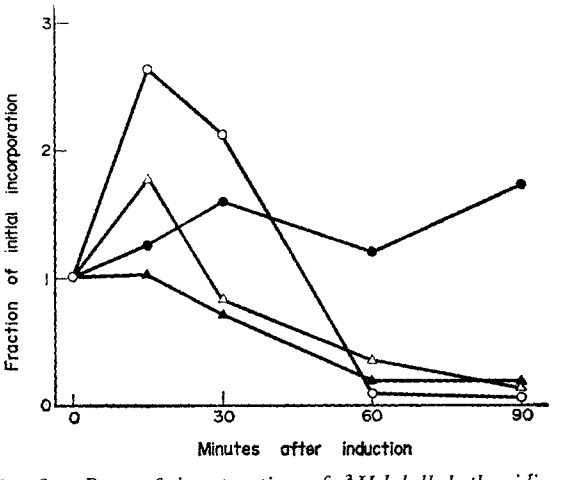


Fig. 8. Rate of incorporation of ^{3}H-labelled thymidine during induction of phage λ in E. coli *PB64 in the presence and absence of the Et derivative (2 μg/ml).*

Four cultures (12 ml) were grown in L broth at 30°C to an absorbance at 650 nm of 0·72. The Et derivative was added to two cultures, and, 5 min later, one drug-treated and one control culture were heated to 42°, while the other two were maintained at 30°C. Immediately after induction and at various times later, samples (1 ml) were removed and incubated with ^{3}H-labelled thymidine (1 μCi/ml) for 1 min. Cold 10% trichloroacetic acid (1 ml) containing unlabelled thymidine (1 mg/ml) was added and the cells were treated as in Fig. 4. The incorporation of ^{3}H-labelled thymidine is expressed as a fraction of the incorporation obtained immediately after the temperature shift, or at the corresponding time for uninduced cultures. The absorbance at 650 nm for each sample at the end of the experiment was: Control uninduced (●—●): 1·71; drug-treated uninduced (▲—▲): 1·23; control induced (○—○): 0·18; drug-treated induced (△—△): 0·88.

The drug did not prevent the increase in thymidine incorporation following induction, but the maximum increase was 30% lower in the drug-treated culture. Thymidine incorporation by the non-induced, drug-treated culture (Fig. 8) was inhibited much more than

would be expected from the results shown in Fig. 4, but projection of the data shown in Fig. 3 suggests that this is a result of the cells reaching stationary phase.

The effect of the drug series on lytic infection by phage lambda

E. coli PB151 was grown in L broth at 30°C to an absorbance at 650 mμ of 0·33 (6×10^6 cells/ml) and various amounts of the drugs Me–Pe were added. Immediately after, a stock solution of phage λ was added at a multiplicity of infection of 3. After 10 min, the cultures were diluted 3-fold in prewarmed L broth containing similar concentrations of drug. The results were qualitatively similar to those obtained following induction. The phage yield obtained in the presence of the Et homolog (2 μg/ml) was 1% of the control culture, which produced 100 phage per bacterium. However, some of the phage assayed could have been those used to infect the culture and the actual extent of inhibition could have been greater. Similar results were obtained with cultures of PB151 grown at 37°C and 42°C. As in the case of induction, infection of *E. coli* in the presence of the Et derivative caused death of the host bacterium.

DISCUSSION

From the series of bisquaternary salts (Me–Pe, Fig. 1) animal screening experiments have selected the Et homolog as the member with optimal antileukaemic properties [2]. In the present study, this compound has been shown to be the most powerful inhibitor of the growth of phage lambda following either induction or infection, whereas the more lipophilic homologs (Bu–Pe) which have little or no antileukaemic activity, have proved to be the most toxic agents for *E. coli*.

In attempting to account for this activity, it should be noted that the drug series was synthesized on the basis of molecular models to fit the DNA molecule [1]. The drugs could bind either to the minor groove of the DNA helix or intercalate between base pairs, in such a way that the two cationic groups interact with phosphate groups on the DNA. All members of the series underwent a bathochromic shift in the absorption maximum (305–315 mμ) in the presence of calf thymus DNA, and also caused (at a concentration of 10 μg/ml) an increase in the denaturation temperature of the DNA solution (Baguley, unpublished results). This demonstrates an interaction between the drugs and DNA.

The drug series could selectively affect the synthesis of DNA or RNA, as a consequence of binding to DNA. However, no selective effect has been observed in *E. coli*. A generalised inhibition of growth has been observed (Fig. 2 and Table 1), but this could equally well result from interference with the function of the cell surface as from interaction with nucleic acids. The rapid lysis of bacteria by the Bu and Pe compounds is consistent with a detergent-like action.

The effects of the Et derivative on growth of phage lambda occur at a drug concentration (1–2 μg/ml) which only slightly inhibits growth of *E. coli* (Figs. 2 and 5), and causes at least 99% inhibition of phage production following either induction or infection. Such inhibition could be accounted for by two main types of explanation.

(a) *A selective effect on phage function.* It is possible that drug interaction with a specific phage protein, or perhaps with a protein-nucleic acid complex, could cause selective inhibition. The lethal effect on the bacteria would then be a result of expression of a phage function [5]. Sly *et al.* [5] concluded from the study of a number of phage mutants that if phage specific DNA synthesis did not occur, then the incorporation of ^{3}H-thymidine into DNA showed an immediate decrease following induction. In view of their results, the experiments here suggest that phage DNA synthesis does occur initially (Fig. 8) although the DNA has not yet been characterized. Since exonuclease activity increases following phage induction, but lysozyme is presumably not produced, the results suggest that some late function of phage lambda is affected. However, the results of "rescue" experiments and those shown in Fig. 6 show that the Et homolog must be present at the time of induction to exert its maximum effect.

(b) *A change in permeability to the drugs following induction.* An increase in permeability at the time of induction could allow a higher intracellular drug concentration to be attained, resulting in generalised inhibition of both phage and bacterium.

Silver [6] has demonstrated that phage T2 has at least two genes affecting the permeability of the host cell to acridines. It is possible that phage λ either itself specifies a gene, or activates a host gene, which changes the permeability of the cell to the compounds in the drug series. Any change in permeability is not due merely to physical damage to the bacterial cell wall, since the drug effects are seen following heat induction as well as following infection with

phage λ. A change in permeability would explain the failure of attempts to "rescue" the bacteria by returning them to the permissive temperature (30°C) following induction in the presence of drug. If cell permeability changed only transiently during induction, then the reason for the necessity for the drug to be present immediately after induction would be clear. A change in permeability of bacterial cells could also explain the premature end of logarithmic phase in drug-treated uninduced cultures (Fig. 2).

Attempts to detect increased uptake of the drug series by *E. coli* following induction have so far been unsuccessful. Ultraviolet absorbance and fluorescence techniques have not been sufficiently sensitive, and the preparation of radioactive drugs is under way.

The interest of this bacterial system is its possible analogy with tumour viruses which are integrated into mammalian cell DNA. It has been shown that low concentrations of the Et homolog (1–2 μg/ml) have little effect on bacteria carrying the phage in a repressed state, but are lethal for bacteria carrying the induced phage. A mixed population of "normal" and "induced" bacteria could be treated to eliminate the proportion producing infective virus. If an analogous situation exists for mammalian cells carrying a tumour virus, then effective chemotherapy should be feasible.

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REFERENCES

1. B. F. Cain, G. J. Atwell and R. N. Seelye. Potential antitumour agents. X. Bisquaternary salts. *J. med. Chem.* **12,** 199 (1969).
2. G. J. Atwell and B. F. Cain. Potential antitumour agents. VI. *J. med. Chem.* **11,** 295 (1968).
3. H. Diggelman and C. Weissmann. Rifampicin inhibits focus formation in chick fibroblasts infected with Rous Sarcoma Virus. *Nature (Lond.)* **224,** 1277 (1969).
4. W. L. Fangman and A. Novick. Mutant bacteria showing efficient utilization of thymidine. *J. Bact.* **91,** 2390 (1966).
5. W. S. Sly, H. A. Eisen and L. Siminovitch. Host survival following infection with or induction of bacteriophage lambda mutants. *Virology* **34,** 112 (1968).
6. S. Silver. Acridine sensitivity of phage T2: a virus gene affecting cell permeability. *J. mol. Biol.* **29,** 191 (1967).
7. C. M. Radding. Regulation of λ exonuclease. I. Properties of λ exonuclease purified from lysogens of λ_{T11} and wild type. *J. mol. Biol.* **18,** 235 (1966).

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Burkitt's Lymphoma: a B or T Cell Tumour?

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Abstract—*Burkitt's Lymphoma (BL) is known to possess some of the features associated with B lymphocytes. Evidence is provided here for the simultaneous possession of T lymphocyte characteristics. Nine of* 25 *tumours* (36%) *responded to* in vitro *PHA stimulation by increased DNA synthesis (measured as tritiated thymidine uptake), and all of* 6 *tumours tested were able to form some spontaneous rosettes with washed sheep erythrocytes, (both T-cell characteristics) as well as with antibody and complement treated sheep erythrocytes (a B-cell characteristic). These findings did not appear to be related to lymphocyte contamination of the tumour cells. The significance of the possession by BL cells of both B and T lymphocyte associated characteristics and the implications of PHA responsiveness are discussed.*

INTRODUCTION

SEVERAL pieces of evidence have indicated that Burkitt's lymphoma (BL) is derived from cells related to B, (bone marrow derived) lymphocytes. These include surface coating with immunoglobulin which is synthesised by the cell [1, 2], the secretion of immunoglobulins by derived cell lines and freshly biopsied BL cells [3, 4], and the production by a cell line (Jijoye) of specific antibody when challenged by an antigen (T2 coliphage) [5]. Recently, several cell lines derived from BL have been shown to form rosettes with sheep red blood cells (SRBC) treated with specific antibody and complement (EAC′) [6], demonstrating the presence of surface receptors for complement. B lymphocytes, but not T (thymic derived) are believed to possess such complement receptors [7, 8]. The present report describes some studies which indicate that BL may also possess some of the characteristics believed to be associated with T-lymphocytes, namely increased DNA synthesis in response to phytohaemagglutinin (PHA) stimulation [9, 10], and the formation of rosettes with washed, untreated SRBC [11, 12, 13]. The experiments have been mainly performed with freshly biopsied tumour cells to avoid the theoretical criticisms that long term cultured cells may not truly representative of the *in vivo* tumour [14], but two human lymphoblastoid cell lines derived originally from BL biopsies were included for comparison.

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MATERIALS AND METHODS

Tumour samples were obtained at the time of surgical biopsy from 27 patients. These tumours were all confirmed both histologically and cytologically as BL. None of the tumours were arising in lymphoid tissue. Most of them were jaw or ovarian tumours, some were mesenteric or retroperitoneal masses, and 2 were subcutaneous masses. In an additional 4 patients tumour cells were obtained from ascitic fluid. Of the 31 tumours, 11 were recurrences following a period of complete remission of at least a month after chemotherapy with cyclophosphamide, and the remainder were initial tumours from untreated patients. The cell lines were Raji and P_3HRI. These were supplied by Dr. R. Herbermann (N.C.I. Bethesda) and were propagated for several weeks in this laboratory prior to use.

Twenty-five tumours were tested for PHA reactivity, and 6 different tumours for EAC and E rosette formation. In addition, in many of the patients, peripheral lymphocyte re-

activity to PHA was assessed on whole blood obtained within 24 hr of the tumour sample. The cell lines were tested both for PHA reactivity and rosette formation.

Preparation and characterisation of tumour cell suspensions

Immediately after removal of the tumour from the patient it was finely minced in RPMI 1640 medium containing 100 U penicillin, 100 μg streptomycin and 0·25 μg fungizone per ml. The resultant cell suspension was filtered through sterile gauze to remove tissue fragments and washed three times in medium (as above). Cell count was performed in a counting chamber, and viability assessed by trypan blue exclusion. To assess lymphocyte contamination of tumour cells, differential counts were performed either on imprint preparations of the original tumour, or on cytocentrifuge (Shandon Ltd.) preparations of the final cell suspension. These preparations were stained with Wright's stain. At least 500 cells were counted, and lymphocyte contamination expressed as a percentage. Where difficulty was experienced in distinguishing small tumour cells from lymphocytes, the cell was counted as a lymphocyte, so that any observer error will tend to increase the percentage lymphocyte contamination.

PHA reactivity

Tumour cell concentration was adjusted to 10^6 viable cells per ml in RPMI 1640 containing 15% foetal calf serum (FCS) and antibiotics. Sextuplet 1 ml culture of unstimulated cells and cells to which 0·05 ml, 0·01 ml and 0·001 ml PHA (Difco, PHA M) per ml had been added, (in some cases the lowest dilution was omitted) were incubated in a 5% CO_2 atmosphere. Triplicate cultures were harvested on days 3 and 5, 18 hr after the addition of 1 μCi tritiated thymidine (H^3Tdr., Sp. activity 5 Ci per mM, Amersham) to each culture. At the time of harvest, cultures were washed in N-saline, and following protein precipitation with 5% trichloroacetic acid (TCA) at 4°C and successive washes in TCA and methanol, the acid insoluble precipitate was solublised in 0·2 ml hyamine hydroxide (Packard) at 56°C for 20 min and mixed with a toluene phosphor scintillation fluid. H^3Tdr uptake was measured in a liquid scintillation spectrometer (Packard, Tricarb) and counting efficiency assessed by the external standard method. Results were expressed as average counts/min (counts/min) per culture for each triplicate, and PHA response as the ratio of the highest counts/min on either day 3 or 5 in a stimulated culture, to the counts/min of the unstimulated culture on the same day (S:U ratio). A ratio of greater than 3 was taken as positive.

PHA reactivity of peripheral blood lymphocytes was assessed by a similar technique. Each culture consisted of 1 ml RPMI 1640 with 15% FCS and antibiotics to which 0·033 ml whole blood [containing 20U preservative free heparin (Pularin) per ml] was added. Triplicate unstimulated and stimulated (0·03, 0·01 and 0·001 ml PHA per culture) cultures were harvested on day 4, 18 hr after the addition of 1 μ Ci H^3Tdr. The technique of harvesting and scintillation counting was as for the tumour cells, except that as a preliminary, red cells were lysed by treatment with 3% acetic acid at 4°C. Results were expressed as the S:U ratio on day 4.

EAC′ rosettes

SRBC were obtained by weekly jugular venepuncture, and preserved in Alsever's solution (1 part blood to 2 parts Alsever's) at 4°C. EAC′ complexes were prepared by washing SRBC × 3 in Hanks balanced salt solution (BSS) and incubating a 5% solution with an equal volume of a 1:2000 dilution of SRBC antistromal antibody (Cordis) for 30 min at 37°C. The cells were then washed × 3 in BSS and resuspended as a 5% solution. Equal volumes of this solution and a 1:80 dilution of freshly obtained human serum as a source of complement, were incubated for 30 min at 37°C, washed × 3 in BSS and finally resuspended as a 2·5% solution in BSS.

The tumour cells were adjusted to 5×10^6 per ml in serum free RPMI 1640 medium containing antibiotics. Equal volumes (0·5 ml) of tumour cells and the final 2·5% EAC′ suspension were mixed in triplicate, centrifuged at 400 rev/min for 10 min and incubated for 30 min at 37°C. Half the supernatant liquid from each tube was carefully aspirated and the pellet gently resuspended in the remainder. A drop was placed on a glass slide with a drop of an isotonic solution of crystal violet to facilitate recognition of tumour cells, and a cover slip placed on top. Three hundred tumour cells per tube were counted, and any with at least 3 adherent SRC's were assessed as "rosettes". The result was expressed as the average percentage of rosette forming cells for the three tubes.

E-rosettes

Equal volumes (0·5 ml) of a 2·5% solution of washed (×3) SRBC and a suspension of tumour cells (as for EAC′ rosettes) were mixed in triplicate, spun down for 10 min at 400 rev/min and incubated for 45 min at 37°C. The mixture was then incubated overnight at 4°C and the percentage of rosette forming cells assessed as for EAC′ rosettes.

Table 1. *Stimulation of Burkitt lymphoma cells by PHA*

Tumour cells S:U ratio	% Lymphocyte contamination	Tumour cell viability (%)	Peripheral blood S:U ratio
No response			
0·2	1·6	78	565
0·3	4	55	61
0·5	—	90	—
0·8	—	82	4
1·0	—	88	—
1·0	—	93	—
1·0	2	55	22
1·2	2·5	98	213
1·2	2	94	4
1·3	1	60	171
1·5	3	86	3·2
1·5	5	92	—
1·7	—	95	21
1·9	—	88	—
1·9	4	86	—
2·5	4	73	150
Positive			
3·3	2	66	28
4·2	0·4	82	—
9·0	1	92	151
9·5	5	72	—
11·5	0·4	80	346
15	2	60	25
18	3	80	250
39	4	98	95
48	6	70	—

RESULTS

PHA stimulation

Nine of the 25 tumour cell preparations assessed for PHA reactivity had an S:U ratio of greater than 3, the highest ratio being 48 (Table 1). Examples of the results of actual experiments are given in Table 2. Several different patterns were seen. Sometimes the H^3Tdr incorporation in some or in all of the stimulated cultures was significantly higher than in unstimulated cultures on both days 3 and 5. Sometimes, particularly where the H^3Tdr incorporation of unstimulated cultures was high, there was no significant difference in stimulated and unstimulated cultures on the 3rd day, but by the 5th day the H^3Tdr incorporation of unstimulated cultures had fallen considerably as spontaneous cell death occurred, whilst that of stimulated cultures remained high or increased. In some experiments recorded as negative, unstimulated counts remained high on day 5, such that the S:U ratio was less than 3. It is possible that these cells were already optimally incorporating H^3Tdr such that no increase could be induced with PHA. In such cases a PHA receptor might have been demonstrable under sub-optimal culture conditions. S:U ratios of greater than 3 were only seen when H^3Tdr incorporation of unstimulated cultures (which varied between 107 and 521,759 counts/min with a median of 5,661 counts/min) was less than 7500 counts/min, although stimulation did not necessarily always occur under these circumstances.

There was no correlation between H^3Tdr incorporation of unstimulated cultures and trypan blue exclusion so that variations in initial tumour cell viability cannot explain the marked variation in DNA synthesis occurring in different tumours during short term culture.

Table 2. *Results of two experiments demonstrating stimulation of Burkitt lymphoma cells by P.H.A.*

	Mean counts per min ± I S.E. M day 3	S:U ratio	Mean counts per min ± I S.E. M day 5	S:U ratio
1. Patient 10				
Control (unstimulated)	100,402 ± 7,128		7,470 ± 1,898	
PHA 0·05 ml	111,169 ± 2,107	1.09	289,988 ± 2,631	38·83
PHA 0·01 ml	101,699 ± 4,488	1·01	361,780 ± 25,601	48·43
PHA 0·001 ml	100,437 ± 9,261	1·00	275,707 ± 24,913	36·90
2. Patient 9*				
Control (unstimulated)	689 ± 15		439 ± 66	
PHA 0·01 ml	530 ± 67	0·76	225 ± 13	0·51
PHA 0·001	20,691 ± 2,947	30·34	17,123 ± 2,467	39·04

*A dilution of 0·05 mls PHA not included in this experiment. Results are expressed as counts/min ± 1 standard error of the mean (S.E.M.).

The degree of lymphocyte contamination of the tumours was variable, but in no case did it exceed 6%. The median was 3%. There was no correlation between the PHA responsiveness of the tumour cell preparations and lymphocyte contamination. In addition there was no correlation between peripheral blood PHA responsiveness and PHA response of the tumour cell preparations. In some experiments a negative tumour cell response to PHA was recorded in the presence of a relatively high percentage lymphocyte contamination and good PHA response of peripheral blood.

Neither of the two lymphoblastoid cell lines tested showed a significant response to PHA stimulation.

Rosette formation

The percentages of EAC′ and E rosettes in the 6 tumours tested and the percentage lymphocyte contamination is shown in Table 3. Since the assessment of rosette formation was a visual test, there was an opportunity to assess the nature of the rosette forming cells. Although adherence of SRBC to the rosetting cell was sometimes so marked that the rosetting cell could not be distinguished, where fewer cells were adherent the cell could frequently be clearly seen to be a tumour cell (in each of the tumours studied). This and the fact that the percentage of rosettes was in almost all cases greater than the percentage of contaminating lymphocytes leaves no doubt that the tumour cells were capable of forming both types of rosette, and that the results could not be explained as rosette formation by contaminating lymphocytes. The P_3HRI cell line did not form either EAC′ or E rosettes, whilst the Raji line formed EAC′ but not E rosettes.

Table 3. EAC′ and E rosette formation in freshly biopsied Burkitt Lymphoma cells, and two cell lines

Tumour	%EAC′	%E	% lymphocyte contamination
AO	31	1	3·3
S	33	5	0·2
AS	11	18	2·0
N*	7	8	2·0
A	9	3	0·4
E	2	6	0·4
P_3HRI	0	0	0
Raji	90	0	0

*Signifies a relapse tumour.
Viability in all tumours was in excess of 70% by trypan blue exclusion.

DISCUSSION

There is no doubt that BL cell lines and freshly biopsied cells have some of the characteristics of B lymphocytes [1–6]. The data presented here suggests that BL, or at least, some BL cells, also manifest some of the characteristics usually associated with T-lymphocytes; namely PHA responsiveness, and E-rosette formation. Although it could be argued that contaminating lymphocytes were responsible for the observed PHA responses with tumour cell preparations, this seems unlikely for several reasons. Firstly, the percentage of lymphocytes in the tumour cell suspension was very small—two responsive tumours contained only 5×10^3 lymphocytes per culture. In chronic lymphocytic leukaemia a much smaller dilution of what have been shown to be normally reactive T lymphocytes by circulating tumour cells leads to a failure to respond to PHA [15]. Secondly, no correlation was observed between the PHA response of the tumour cell preparations and the degree of lymphocyte contamination, even when the response to PHA of peripheral blood lymphocytes was taken into account.

Since BL contains histiocytic cells (which give rise to the characteristic "starry sky" histological appearance) the lack of correlation cannot be attributed to absence of glass-adherent macrophages from the tumour cell suspensions.

It seems likely then, that the tumour cells themselves were responding to the PHA stimulus, and any contribution by contaminating lymphocytes was insignificant. Autoradiographic, or morphological assessment of PHA response would not be of help in verifying this because of the close resemblance of transformed lymphocytes to tumour cells, but such a finding is not unprecedented, since other lymphoid tumours have been described in which there is a similar *in vitro* response to PHA [16]. E rosette formation—a quite different T cell characteristic—provides additional evidence that some BL cells possess at least some of the features of T lymphocytes.

The possession by BL of both B and T lymphocyte characteristics raises questions concerning the cell of origin of BL, and also the relationship between B and T lymphocytes in terms of lymphoid cell differentiation. There are several possible explanations for this finding: (1) each tumour may contain both cell types by virtue of a polyclonal origin; (2) some cells are capable of simultaneously expressing both T and B properties; (3) differentiation from one cell type to the other is occurring within the

tumour cell population. A polyclonal origin of BL would be the simplest explanation, but evidence against this is provided by studies of iso-enzyme phenotypes [17] (glucose-6-phosphatase and phosphoglucomutase), although this is not entirely supported by data concerning the secretion of immunoglobulins, since freshly biopsied tumours have been shown to secrete more than one class [4]. If BL is monoclonal, the expression of both B and T characteristics is not so readily explained. Nonetheless, since both types of lymphocyte must ultimately be derived from a single precursor cell, it is not impossible to envisage several circumstances under which this may arise. Firstly, the tumour may have developed from the precursor cell itself, when each cell could potentially express either or both sets of characteristics, perhaps depending upon the degree of differentiation. Secondly, derepression may have occurred in a more differentiated cell (which would normally express either B *or* T characteristics) allowing the usually unexpressed receptors to become apparent; and thirdly, membrane changes associated with the malignant transformation may have unmasked a cryptic receptor site, achieving the same effect.

Further studies are required to determine whether the same cells may manifest both B and T characteristics simultaneously, or whether there are sub-populations of cells. However there is some evidence that T cells do carry surface immunoglobulin in very small quantities, [18, 19] and a thymic lymphoma of AKR mice has been described in which this is considerably increased [20], suggesting that in some malignant lymphomas, the dividing line between B and T cells may be much less distinct than in normal cells.

Since in the present study different tumours varied markedly in their ability to express B and T cell characteristics, the possibility that the qualitative and quantitative differences in expression are clinically significant, should be considered, although no such correlations are apparent in the patients described here.

If tumour cells respond to PHA stimulation by increased DNA synthesis, a similar response may occur *in vivo* with non-specific mitogens such as immune complexes [21], which could therefore function in this system not only as blocking factors [22], but also as true enhancing factors. Immune complexes quite unrelated to the tumour such as those which occur in parasitic diseases [23] could also have clinical significance. Certainly malaria has been implicated in the aetiology of a mouse lymphoma [24] and of BL [25, 26]. Antibody/antigen complexes, or antibody alone have therefore a potential role in producing the very rapid doubling time of BL [27] and the stimulatory effect of BL serum on the proliferation of BL cells which has been observed *in vitro* [28, 29].

Clearly, the nature of the relationships between the surface membrane of lymphoid neoplasms and normal lymphocytes is worthy of further study. Not only may some insight be gained into pathogenesis, but information concerning factors which effect the proliferation of tumour cells, and therefore may be of prognostic importance may be obtained. Further, characterisation of surface features such as the possession of B or T cell receptors may be of value in the classification of lymphomas.

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REFERENCES

1. E. Klein, G. Klein, J. Nadkarni, S. Nadkarni and C. Clifford, Surface IgM-Kappa specificity of a Burkitt lymphoma cell *in vivo* and in derived culture lines. *Cancer Res.*, **28,** 1300, 1968.
2. J. Nadkarni, S. Nadkarni, P. Clifford, G. Manolov, E. Fenyo and E. Kein, Characteristics of new cell lines derived from Burkitt lymphomas. *Cancer (Philad.)*, **23,** 64, 1969.
3. E. Klein, R. van Furth, B. Johansson, F. Emberg and P. Clifford, Immunoglobulin synthesis as cellular marker of malignant lymphoid cells. In Proc. *Cambridge Symposium on Oncongenesis and Herpes Type Viruses* 1971.
4. R. van Furth, H. Gorter, S. Nadkarni, J. Nadkarni, E. Klein and P. Clifford, Synthesis of immunoglobulins by biopsied tissues and cell lines from Burkitt's lymphoma. *Immunology* **22,** 847, 1972.
5. H. Kamei and G. E. Moore, Production of Phage-neutralizing activity *in vitro* by cells derived from Burkitt's lymphoma. *Nature (Lond.)* **215,** 860, 1967.
6. E. M. Shevach, R. Herberman, M. Frank and I. Green, Receptors for complement and immunoglobulin on human leukaemic cells and human lymphoblastoid cell lines. *J. clin. Invest.* **51,** 1933, 1972.

7. C. Bianco, R. Patrick and V. Nussenzweig, A population of lymphocytes bearing a membrane receptor for antigen–antibody–complement complexes. *J. exp. Med.* **132,** 702, 1970.
8. V. Nussenzweig, C. Bianco, P. Dukor and A. Eden, Receptors for C 3 on B lymphocytes. Possible role in the immune response. In *Progress in Immunology* (Edited. by B. Amos) Academic Press, New York, 1971.
9. M. J. Doenohoff, A. J. S. Davies, E. Leuchars and V. Wallis, The thymus and circulating lymphocytes of mice. *Proc. R. Soc. Ser. B.* **1976,** 69, 1970.
10. G. Janossy and M. F. Greaves, Lymphocyte activation. II Discriminating stimulation of lymphocyte subpopulations by phytomitogens and heterologous antilymphocyte sera. *Clin. exp. Immun.* **10,** 525, 1972.
11. J. Jondal, G. Holm and H. Wigzell, Surface markers on human T and B lymphocytes. A large proportion of lymphocytes forming non-immune rosettes with sheep red blood cells. *J. exp. Med.* **136,** 207, 1972.
12. W. Lay, II, N. F. Mendes, C. Bianco and V. Nussenszweig, Binding of sheep red blood cells to a large population of human lymphocytes. *Nature (Lond.)* **230,** 531, 1971.
13. J. Wybran, M. C. Carr and H. H. Fudenberg, The human rosette-forming cell as a marker of a population of thymus-derived cells. *J. clin. Invest.* **51,** 2537, 1972.
14. P. J. Fialkow, G. Klein, E. R. Giblett, B. Gothoskar and P. Clifford, Foreign cell contamination in Burkitt tumours. *Lancet* **i,** 883, 1971.
15. J. Wybran, S. Chantler and H. H. Fudenberg, Isolation of normal T cells in chronic lymphatic leukaemia. *Lancet* **i,** 126, 1973.
16. J. Brody, Augmentation of leukaemic lymphocyte reactivity to phytohaemagglutinin by salivary receptor. *Blood* **40,** 70, 1972.
17. P. J. Fialkow, G. Klein, S. M. Cortler and P. Clifford, Clonal origins for individual Burkitt tumours. *Lancet* **i,** 374, 1970.
18. A. D. Bankhurst, N. L. Warner and J. Sprent, Surface immunoglobulin on thymus and thymus-derived lympoid cells. *J. exp. Med.* **134,** 1005, 1971.
19. J. J. Marchalonis, J. L. Atwell and R. E. Cone. Isolation of surface immunoglobulin from lymphocytes from human and murine thymus. *Nature New Biol.* **235,** 240, 1973.
20. H. M. Grey, S. Colon, P. Campbell and E. Rabellino, Immunoglobulins on the surface of lymphocytes. V. Quantitative studies on the question of whether Immunoglobulins are associated with T cells in the mouse. *J. Immunol.* **109,** 776, 1972.
21. G. Moller, Induction of DNA synthesis in normal human lymphocyte cultures by antigen–antibody complexes. *Clin. exp. Immunol.* **4,** 65, 1969.
22. R. W. Baldwin, M. R. Price and R. A. Robins, Blocking of lymphocyte mediated cytotoxicity for rat hepatoma cells by tumour-specific antigen/antibody complexes. *Nature New Biol.* **238,** 185, 1972.
23. V. Houba and A. C. Allison, M-antiglobulins (Rheumatoid-factor-like globulins) and other gamma globulins in relation to tropical parasitic infections. *Lancet* **i,** 848, 1966.
24. N. Wedderburn, Effect of concurrent malarial infection on development of virus-induced lymphoma in Balb/c mice. *Lancet* **ii,** 1114, 1970.
25. D. P. Burkitt, Etiology of Burkitt's Lymphoma—an alternative hypothesis to a Vectored Virus. *J. nat. Cancer Inst.* **42,** 19, 1969.
26. J. L. Ziegler, A. Z. Bluming, R. H. Morrow, M. H. Cohen, E. H. Fife, J. R. Finnerty and R. Woods, Burkitts lymphoma and malaria. *Trans. Roy. Soc. Trop. Med. Hyg.* **66,** 285, 1972.
27. U. Iversen, O. H. Iversen, A. Z. Bluming, J. L. Ziegler, and S. Kyalwazi, Cell kinetics of African cases of Burkitt lymphoma. *Europ. J. Cancer* **8,** 305, 1972.
28. B. O. Osunkoya, In *Burkitt's Lymphoma,* (Edited by D. P. Burkitt and D. H. Wright), p. 134. E. & S. Livingstone, Edinburgh, 1970.
29. I. T. Magrath, Unpublished data.

Recently, evidence for a small population of human peripheral blood lymphocytes bearing both T and B lymphocyte markers has been published.

H. B. Dickler, N. F. Adkinson, Jr., W. D. Terry. *Nature (Lond.)* **247,** 213–215. 1974.

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Factor Time for Active Immunotherapy After Cytoreductive Chemotherapy*

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Abstract—*In the treatment of leukaemia E♂ G2 in F1(C_{57}B1/6 × DBA/2) mice by a cytoreductive chemotherapy with procarbazine followed by a specific active immunotherapy by B.C.G., the time interval between the end of chemotherapy and the beginning of immunotherapy is a very critical factor. The sooner the immunotherapy, the better the results, a delay of more than* 48 *hr compromized greatly the chances of cures.*

INTRODUCTION

NON-SPECIFIC immunotherapy given before [1] or after [2] the injection of syngeneic murine leukaemic cells is able to control the proliferation of these cells and to assure the survival of mice.

Immunotherapy has only been found to be effective if the number of malignant cells is less than a critical maximum (10^4 cells for the murine E♂ G2 leukaemia, 10^5 for the 1210 murine leukaemia) and it is only effective when it is not followed by cytostatic chemotherapy [3, 4].

The experimental models differ notably from the clinical situation encountered in man. The immunotherapy is tested alone in normal animals receiving a graft of leukaemic cells, but whose immune defences have not been altered by immunosuppressive cytostatic agents [5], nor by the late effects of the leukaemic population *per se* [6, 7].

Active immunotherapy is also somewhat effective in the treatment of the "spontaneous" leukaemia of AkR mice, given alone, or after cytostatic chemotherapy, but these experimental procedures are long and difficult [8].

We have shown that the protocol of therapy by two steps, firstly reducing the number of leukaemic cells, given a too great number to be cured by immunotherapy alone, secondly eradicating the remaining leukaemic cells by active immunotherapy is very effective [9], but the course of chemotherapy has also an immunosuppressive action. This immunosuppression is long-lasting and cannot be prevented by a first preparation of the mice by non-specific immunotherapy [7].

It seems therefore in mice that the best protocol is to give immunotherapy only after stopping any immunosuppressive chemotherapy, but the next question seems to know what is the best moment to give non-specific immunotherapy, as we have to consider that the possibilities of the treated mice to react to non specific immunotherapy will improve with time after stopping the chemotherapy and even could have an "immunological rebound" but also that the proliferation of the remaining leukaemic cells can overflow the maximum possibilities of immune defences and will *per se* diminish these immune mechanisms [7].

We have tried to answer these questions using a compatible leukaemia, E♂ G2 in F1 (C_{57}B1/6 × DBA/2) mice, procarbazine as a cytoreductive chemotherapy, B.C.G. as non-specific active immunotherapy.

MATERIAL AND METHODS

Sixty F1(C_{57}B1/6 × DBA/2) male mice aged 3 months were given 10^5 compatible E♂ G2 leukaemic cells intraperitoneally on day 0,

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they were divided into 6 groups of 10 mice. The mice in Group I received no treatment, the mice of the 5 other groups received from day 4 to day 9 inclusive, 123 mg/kg procarbazine per day intraperitoneally, that is one sixth of LD 50 at 6 days (Garattini, personal communication).

The mice of Group II received no other treatment, the mice of the other groups received intravenously living B.C.G. (1 mg dry weight per mouse) obtained from Institut Pasteur on day 10 for Group III, day 11 for Group IV, day 12 for Group V, day 14 for Group VI.

The animal survival was studied up to 180 days, the presence of leukaemia was checked macroscopically in any dead mouse.

RESULTS

The results are summarized in Table 1.

Procarbazine alone at this dosage gave a considerable increase in the animals' surviving time did but not produce any cure. The addition of a secondary B.C.G. therapy did produce cures but, there is a striking difference in the results of the two groups receiving B.C.G. one or two days after stopping procarbazine, 7 mice cured out of 10 in each group, and the results of the two last groups receiving B.C.G. after a longer gap after stopping chemotherapy, 3 or 5 days, one mice cured out of 10 in each group. If we pool the results of Group III and IV (early B.C.G.), on one hand, and of Groups V and VI (late BCG) on the other hand, the difference is highly significative ($\chi^2_{\text{corr. Yates}} = 15,36$ for 1 dl, S for $P < 0{\cdot}001$).

The last mice treated with B.C.G. to die of leukaemia, died on day 66.

DISCUSSION

Our results show that the time interval between the end of cytoreductive chemotherapy and starting active immunotherapy is a very critical factor in the end result of the treatment of a compatible leukaemia. The optimal time interval can vary according to the type of malignancies treated and the type of chemotherapy used.

Currie and Bagshawe [10] using a slowly growing fibrosarcoma, grafted subcutaneously, treated it by cyclophosphamide followed by heat killed *Corynebacterium parvum*. The time interval between chemotherapy and immunotherapy was also critical; but the optimal interval was long, 12 days. Obviously, the type of tumor, the site and the kinetics of its growth is of great importance.

Pearson and Chirigos [11] treated a murine leukaemia, LSTRA, by BCNU followed by a *Corynebacterium granulosum* or B.C.G., the optimal time interval was short for *Corynebacterium granulosum*, 3 days, but unusually long for B.C.G., 12 days, the results being obtained for animals in relapse, some cures are obtained for shorter intervals. It is possible that the long lasting toxicity of BCNU can play a role to explain the

Table 1. Effects of a non-specific active immunotherapy by B.C.G. preceded by a cytoreductive chemotherapy in F1(C_{57}B1/6 × DBA/2) mice given 10_5 compatible E♂ G2 leukaemic cells intraperitoneally according to the gap between chemotherapy and immunotherapy

Group	No. of mice	The mice died of leukaemia	Mean, median and limits of survival time in days of mice that died of leukaemia
I	10	10	20·5d – 20d (18d – 24d)
II	10	10	39·9d – 34d (26d – 69d)
III	10	3	36d – 41d (27d – 42d)
IV	10	3	30·3d – 28d 26(d – 37d)
V	10	9	46·1d – 45d (31d – 62d)
VI	10	9	39·5d – 34d (28d – 66d)

Group I: Not treated
Group II: Procarbazine d4 to d9
Group III: Procarbazine d4 to d9, B.C.G. d10
Group IV: Procarbazine d4 to d9, B.C.G. d11
Group V: Procarbazine d4 to d9, B.C.G. d12
Group VI: Procarbazine d4 to d9, B.C.G. d14.

discrepancies of these results and of our data.

Our results with B.C.G. on a rapidly growing leukaemia, however, point the danger of waiting to start active immunotherapy. Procarbazine is a very potent immunodepressive agent in mice [12] and the immunodepression evolved by procarbazine is long lasting [13], but B.C.G., 24 hr after stopping a six day course of procarbazine corrects partly this immunodepression (Amiel, not published).

It seems therefore that in the clinical trials on acute lymphoid leukaemia, whose proliferation is a rapid one, it is worthwhile to start active immunotherapy very soon after stopping chemotherapy, and not to wait for the complete recuperation of normal immune reactions or even for the immunological rebound.

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. Mathe, Immunothérapie active de la leucémie L1210 appliquée apres la greffe tumorale. *Rev. franç. Etud. clin. biol.* **13,** 981 (1968).
3. J. L. Amiel and M. Berardet, Essais de traitement de la leucémie Eo G2 associant chimiothérapie et immunothérapies actives non spécifique et spécifique. *Rev. franç. Etud. clin. biol.* **14,** 685 (1969).
4. G. Mathe, J. L. Amiel, L. Schwarzenberg, M. Schneider, M. Hayat, F. de Vassal, C. Jasmin, C. Rosenfeld and P. Pouillart, Preliminary result 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.* **16,** 216 (1971).
5. J. L. Amiel, M. Sekiguchi, G. Daguet, S. Garattini and V. Palma, Etude de l'effet immunodépresseur des composés chimiques utilisés en chimiothérapie anticancéreuse. *Europ. J. Cancer* **3,** 42 (1967).
6. M. Schneider and J. F. Dore, Effet de l'injection d'un virus leucémogéne murin (Friend) sur les défenses immunitaires de souris sensibles et résistantes à ce virus. *Rev. franç. Etud. clin. biol.* **14,** 1010 (1969).
7. J. L. Amiel and M. Berardet, Attempts to prevent acquired immunological insufficiencies in mice by Bacillus Calmette-Guérin. *Experientia* **27,** 941 (1971).
8. G. Mathe, O. Halle-Pannenko and C. Bourut, Active immunotherapy of AkR mice spontaneous leukemia. *Exp. Hematol.* **1,** 110 (1973).
9. J. L. Amiel et M. Berardet, An experimental model of active immunotherapy preceded by cytoreductive chemotherapy. *Europ. J. Cancer* **6,** 557 (1970).
10. G. A. Currie and K. D. Bagshawe, Active immunotherapy with *Corynebacterium parvum* and chemotherapy in murine fibrosarcomas. *Brit. med. J.* **1,** 541 (1970).
11. J. W. Pearson, G. R. Pearson, W. T. Gibson, J. C. Chermann and M. A. Chirigos, Combined chemo-immunostimulation therapy against murine leukemia. *Cancer Res.* **32,** 904 (1972).
12. J. L. Amiel, C. Brezin, M. Sekiguchi, A. M. Mery, B. Hoerni, S. Garattini, G. Daguet and G. Mathe, Dépression des réactions immunitaires par une méthylhydrazine. *Rev. franç. Etud. clin. biol.* **9,** 636 (1964).
13. J. L. Amiel and M. Berardet, 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).

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Experience with 4'-Demethylepipodophyllotoxin 9-(4,6-o-2-thenylidene-beta-D-glucopyranoside); VM-26; NSC-122819 in the Treatment of Malignant Lymphosis†

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Abstract—*Twenty-three patients, 16 with Hodgkin's Disease, 6 with lymphosarcoma and one with reticulum cell sarcoma were treated with the podophyllotoxin derivative 4'-demethylepipodophyllotoxin 9-(4,6-o-2-thenylidene-beta-D-glucopyranoside); (VM-26; NSC-122819). All patients had far advanced, stage IIIB or IV disease. With the exception of 4 patients, all had received prior therapy. An initial dosage schedule of 40 mg/M² three times weekly was reduced to 50 mg/M² twice weekly in the last 17 patients. The overall objective tumor response rate was 57%. In the patients with Hodgkin's Disease it was 69% including 2 complete remissions. The median remission duration for Hodgkin's Disease was 128 days (26 to 516+ days). The non-Hodgkin lymphoma remissions were shorter with a median of 33 days. The major toxic effect encountered was leukopenia with a lesser degree of thrombocytopenia. This was seen in all but 3 patients. It occurred rapidly, but was also quickly reversible. Over half the patients experienced nausea and vomiting. This limited treatment in only one patient. The value of this compound as monotherapy in malignant lymphomas has been proven. A trial of its use in combination chemotherapy is recommended.*

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INTRODUCTION

PODOPHYLLIN has been used as a cytostatic agent for over thirty years. Originally it was used in the topical treatment of *condylomata acuminata* [1]. Ten years ago two podophyllotoxin derivatives were synthesized in the Sandoz research laboratories and made available for clinical trial [2–5]. Although some clinical usefulness could be demonstrated with these compounds (SPI-77, NSC-72274 and SPG-827, NSC-42076), they were never widely used. The availability of more active and better tolerated cytostatic agents contributed to their disuse. Further development at the Sandoz research laboratories resulted in the new podophyllotoxin derivative epipodophyllotoxin, 4'-demethyl-9-(4,6-o-2-thenylidene-beta-D-glu-

copyranoside) (VM-26; NSC-122819). This new substance has a molecular weight of 656·7. The empirical formula is $C_{32}H_{32}O_{13}S$.

Fibroblast culture studies showed an immediate spindle poison effect with arrest of mitosis in metaphase. However, upon further incubation with the drug cells no longer entered the mitotic phase [6]. This effect is irreversible and differs distinctly from that of other spindle poisons such as colchicine and vinblastine. Subsequent studies place the major drug effect in G_2[§]. ^3H thymidine incorporation into DNA of P-815 mastocytoma cells was inhibited over 50% in drug concentrations of 0·1 μg/ml. Uridine and leucine uptake was minimally, if at all, affected [7]. The drug was shown to be effective in Walker carcinosarcoma 256, Sarcoma-180 and Sarcoma-37. Mice inoculated with L_{1210} leukemia cells showed a definite response to treatment with VM-26 [6]. These animal studies indicated a superiority of intermittent over daily treatment schedules in all tumor systems tested. Toxicology studies showed good tolerance at therapeutic drug dosage levels.

Muggia *et al.* found a dose of 67 mg/M^2 weekly to be tolerable [8]. A clinical trial by Dombernowsky *et al.* established the therapeutic dose at 1–3 mg/kg twice weekly [9]. He also reported responses in patients with malignant lymphomas as did Trempe *et al.* [10], and the EORTC Group [11].

MATERIAL AND METHODS

In fall of 1971 VM-26 was made available to us for clinical trial. It was supplied in 5 ml ampules containing 50 mg 4′-demethyl-9-(4,6-o-2-thenylidene-beta-D-glucopyranoside) with 2500 mg cremophor, 300 mg dimethylacetamide and ethanol as diluents. A pilot study at the Oncology Service, Medical Polyclinic of the University of Basel established a tolerable and effective dosage schedule of 50 mg/M^2 given at 3–4 day intervals twice weekly as a 30–45 min infusion in 250–500 ml physiologic saline or 5% glucose in water. An initial dosage schedule of 40 mg/M^2 given three times weekly had produced rather severe leukopenia and a lesser degree of thrombocytopenia. In January 1972 the Swiss Cooperative Group for Clinical Cancer Research (SAKK) instituted a phase II protocol for further study of VM-26 in patients with malignant lymphoma. The following report includes 10 cases of Hodgkin's Disease from the Basel Polyclinic and a further 6 cases from the SAKK protocol. Also included are 6 cases of lymphosarcoma and 1 case of reticulum cell sarcoma from the SAKK study.

Of the 23 patients treated, 15 were male and 8 were female. The median age was 51 with an age range of 22–75 years. The group of Hodgkin's Disease patients contained 13 patients with stage IV disease and 3 with stage III B disease. Three patients were previously untreated, 5 had had previous radiotherapy, 2 had prior chemotherapy and 6 had both prior radiotherapy and chemotherapy (see Table 3). The one patient with reticulum cell sarcoma had pulmonary involvement. Of the 6 cases with lymphosarcoma, 3 had bone marrow infiltration, 2 had massive skin infiltrates and one had probable liver involvement. One patient with lymphosarcoma also had chronic myelogenous leukemia. After 3 weeks of treatment without improvement he was taken off protocol. New bone marrow studies at that time were compatable with a blastic crisis of chronic myelogenous leukemia. All patients had definite clinically measurable progressive disease at the time of entry into the study, and the disease had been histologically proven. Patients having received radiotherapy in the previous 4 weeks or cytostatic treatment within 2 weeks were not eligible for study. Also, patients with a leukocyte count below 4000 or platelets below 100,000 which were not considered to be diseases related, or a BUN over 30 mg% were ineligible. The patients with Hodgkin's Disease had a median duration of disease of 25 months from the time of diagnosis to the onset of VM-26 treatment. The range was 0–137 months.

The first 6 patients in the pilot study at Basel were initially treated three times weekly with a dose of 40 mg/M^2. The remaining 17 patients were started on therapy with a dose of 50 mg/M^2 twice weekly. Dosage was reduced, or entirely omitted according to the degree of leukopenia or thrombocytopenia observed (see Table 2). If no tumor response or toxicity was noted after 3 weeks of treatment (6 infusions) the protocol allowed for a dosage increase of 20 mg/M^2 every subsequent 2 weeks. Therapy was continued for at least 8 weeks if hematologic toxicity allowed. Patients showing no objective tumor regression at the end of this time despite having reached toxicity were taken off study. Patients with definite objective tumor regression were continued on maintenance treatment with one infusion weekly.

The WBC was controlled twice weekly (prior to each infusion). Platelets, hemoglobin and urinary sugar content were determined weekly.

[§]Personal communication, Sandoz AG Basel.

Symptoms with particular attention to pain, gastrointestinal, cardiovascular and neurologic complaints as well as weight and tumor measurements were recorded weekly. A differential blood count, BUN, alkaline phosphatase, SGOT, SGPT, blood sugar and a complete urinalysis were done every two weeks. Chest X-rays were made at least every four weeks, or as needed for evaluation. Baseline bone marrow, electrophoretic and skeletal survey studies were repeated at the end of the study. If they were initially abnormal, more frequent controls were made.

RESULTS

The shortest period of treatment was 14 days. The longest was over 546 days with a median of 58 days. Of the six patients initially treated three times weekly, the dosage had to be reduced in all six, and therapy had to be temporarily interrupted in four because of marrow depression. Two of these patients were subsequently continued on a twice weekly treatment schedule. In the 17 patients initiated on the twice weekly regime, the dose had to be reduced in 13 and temporarily omitted in 8 of these due to marrow toxicity. One patient had treatment interruption without prior dosage reduction. Another patient was taken off study after 50 days because of nausea and vomiting without ever having become leukopenic. The patient in blastic crisis of chronic myelogenous leukemia was also taken off treatment after 21 days with increasing thrombocytopenia. This was probably not drug related. Dosage was increased in four patients. In all four, this increase followed an earlier dosage reduction. Only one patient was taken off study with progressive disease without any toxicity after 32 days of therapy. Thus, with the exception of one patient with rapidly progressive Hodgkin's Disease after 32 days of treatment and the patient with simultaneous lymphosarcoma and chronic myelogenous leukemia in blastic crisis, all patients demonstrated some degree of drug toxicity.

Tumor response was judged by the reduction in size of measurable tumor masses. Complete remission signifies disappearance of all tumor manifestations. Partial remission I is a 50–90% decrease in measurable tumor parameters. Partial remission II is a 25–50% decrease in measurable tumor. No change denotes neither tumor regression or progression. Progressive disease indicates an increase in tumor size of 25% or more under treatment.

The three times weekly treatment schedule produced one complete, three partial I, and one partial II remissions out of six patients so treated. Only one case showed no change (see Table 1). Of the ten patients with Hodgkin's

Table 1. *Dosage Adjustment*

Leukocytes	Platelets	% dose
Over 5,000	Over 100,000	100
4,000–5,000	75,000–100,000	75
3,000–4,000	50,000–75,000	50
Under 3,000	Under 50,000	0

Disease treated twice weekly, there was one complete remission, two partial I and one partial II remissions. Four patients showed no change and two had progressive disease. Two patients with lymphosarcoma attained a partial I remission and one a partial II remission. One patient showed no change. In two patients the disease was progressive. The one patient with reticulum cell sarcoma had a partial II remission.

The onset of tumor response, when it occurred, was rapid in the non-Hodgkin lymphomas with a median of 10 days (9, 10, 10, and 29 days respectively). The initial response in the Hodgkin patients was seen somewhat later. The median was 24 days. Patients treated three times weekly did not respond more rapidly than those treated twice weekly (median 28 days vs 23 days). The relationship between response to VM-26 therapy and previous treatment is given in Table 3. As could be expected, patients without prior treatment did well. All four attained either complete or good partial remissions.

Remission duration for the Hodgkin's Disease patients ranged from 26 to 516+ days with a median of 128 days. Median remission duration for the twice weekly treated patients was 110 days (four cases; 47 to 516+ days). For the patients treated three times weekly it was 244 days (five cases; 52 to 395 days). The non-Hodgkin lymphoma remissions ranged from 26 to 60 days with a median of 33 days.

Toxicity

Leukopenia was the most common toxic effect recorded. It was seen to some degree in all but 3 patients (see Table 4). Seven of the 23 patients had a white blood count below 2000, and five a count below 1000. Thrombocytopenia below 50,000 was encountered seven

Table 2. VM-26; objective therapy results

		Total No. cases	Complete remiss.	Partial remiss. I (50–90%)	Partial remiss. II (25–50%)	No change	Progress disease
Hodgkin's Disease	2 times weekly dosage	10	1	2	1	4	2
	3 times weekly dosage	6	1	3	1	1	0
Lymphosarcoma		6	0	2	1	1	2*
Reticulum cell sarcoma		1	0	0	1	0	0

*I case with simultaneous blast crisis of chronic myelogenous leukemia.

Table 3. Results of VM-26 treatment according to previous therapy

		No previous therapy	Radiotherapy	Chemotherapy	Radiotherapy and Chemotherapy	Total
Hodgkin's Disease	Complete	1	0	0	1	2
	Partial remission I	2	2	0	1	5
	Partial remission II	0	0	1	1	2
	No change	0	2	0	3	5
	Progressive disease	0	1	1	0	2
Lympho Reticulo Sarc.	Complete remission	0	0	0	0	0
	Partial remission I	1	0	0	1	2
	Partial remission II	0	0	1	1	2
	No change	0	0	0	1	1
	Progressive disease	0	0	0	2	2
Total		4	5	3	11	23

Table 4. Hematologic toxicity with VM-26

		No. Pat.	Leukocytes					Platelets				
			0	1	2	3	4	0	1	2	3	4
Hodgkin's Disease	3 times weekly therapy	6	0	1	0	4	1	1	2	2	0	1
	2 times weekly therapy	10	2	2	2	2	2	5	2	1	0	2
Hodgkin total		16	2	3	2	6	3	6	4	3	0	3
Lympho-retic.-Sarc.		7	1	2	1	1	2	1	0	1	1	4
Over-all total		23	3	5	3	7	5	7	4	4	1	7

Table 4 key:

Leukocytes	Platelets
1—3,000–4,000	100,000–150,000
2—2,000–3,000	75,000–100,000
3—1,000–2,000	50,000–75,000
4—under 1,000	under 50,000

times. This appeared to occur more frequently in the non-Hodgkin lymphoma patients (4 of 7 cases). Two of these four had definite bone marrow infiltration with tumor cells. Five patients were considered to have disease related bone marrow insufficiency upon entry into the study. Five had platelet values between 18,000 and 99,000. Three had a WBC between 1500 and 3300.

Leukopenia appeared rapidly in this group of patients. It was seen in the Hodgkin patients after a median of 11·5 days of treatment, causing the first dose reductions at that time. No significant difference was noted between patients on the twice and three times weekly schedules (10·5 and 12 day medians respectively). The amount of previous therapy also did not seem to influence the time interval for the development of leukopenia. The four previously untreated cases became leukopenic on the 9th, 12th, 13th and 25th days of therapy. WBC recovery was rapid and occurred within 14 days after drug cessation. Thrombocytopenia never occurred without simultaneous leukopenia and also recovered rapidly. No deaths were attributable to marrow depression although one patient was placed in reverse isolation for two weeks.

Only three patients had a relative and absolute lymphocytopenia appear under treatment. One of these was the patient with chronic myelogenous leukemia in blastic crisis.

A fall in hemoglobin levels of 2·0 g% or more was seen in 11 patients. With three exceptions all these patients had disease related anemia at the onset of treatment. In three cases the hemoglobin levels spontaneously returned to normal under continued therapy following an initial fall. In two others with anemia the hemoglobin became normal under treatment as they went into remission.

The most frequent form of non-hematologic toxicity noted was nausea and vomiting. This occurred during or shortly after the VM-26 infusions in 12 patients. It was considered severe in three cases, moderate in another three and mild in six. This effect was almost universally controllable with antiemetics. Treatment was stopped for this reason in only one patient after 50 days. Eight patients had hair loss during therapy. This was severe in four, moderate in three patients and mild in one patient. Microscopic hematuria was recorded in three patients. This developed during thrombocytopenia and was therefore, at least indirectly, drug related. This complication was always of short duration and subsided spontaneously after discontinuing treatment. A moderate rise in SGPT levels to between 20 and 40 I.U. was seen in four cases. In all, it may have been related to the drug, but it did not rise further despite continued VM-26 treatment and receded in three.

Other drug related abnormalities in liver function or in renal function were not noted. Phlebitis or local irritation at the site of injection was not encountered. Cardiovascular or neurologic toxicity was also not seen. The occasional complaints of weakness and tiredness were difficult to separate from symptoms due to the underlying disease process.

DISCUSSION

A tumor response in 57% of the patients treated is encouraging. The response rate among the 13 cases with Hodgkin's Disease was 69%. The quality of response in the Hodgkin group was also very good and included two complete remissions. Also, the remission duration for the Hodgkin patients with a median of over four months was very gratifying. One of these patients is still in remission after one and a half years. As almost half these responders had received previous chemotherapy, and all demonstrated progressive disease at the time of entry into the study, the value of VM-26 as a monotherapy in this disease seems to have been definitely established. Vincaalkaloid drugs were a part of the prior chemotherapy in all cases. The different mechanism of action of VM-26 from the vincaalkaloides and other spindle poisons as demonstrated in cell culture [6] would also seem to be of clinical value and significance.

Remissions in the non-Hodgkin malignant lymphomas were of short duration. With one exception, all had had prior chemotherapy and were in a far advanced stage of disease. Even a short term response in over 50% of such patients indicates definite drug activity and usefulness.

The hematologic toxicity encountered was definitely tolerable and rapidly reversible. Although the number of cases is small, it appears that the remission duration in Hodgkin's Disease patients initially treated three times weekly was longer than that for the patients treated twice weekly. Also, the quality of remission as well as the response rate (67%) seems better in the group treated three times weekly. This superiority was, however, clearly associated with a greater degree of marrow toxicity. Five of the six patients had leukopenia below 2000. The onset of leukopenia was rapid in both treatment groups. This was similar in the lymphoma patients reported by Dombernowsky *et al.* [9]. The almost constantly

observed phlebitis and local irritation at the site of injection reported by these authors [9] was not encountered in our patients. The formulation of VM-26 used in our study consisted of ampules containing 50 mg of the drug with 2500 mg cremophor, 300 mg dimethylacetamide and ethanol as solvents. This was twice the amount of active drug in an equal amount of solvent as that used by Dombernowsky *et al.* This would confirm their suspicion that the local toxicity was due to the solvents.

It is felt that VM-26 is of definite clinical usefulness in the treatment of patients with Hodgkin's Disease and other malignant lymphomas. Its value as monotherapy has been demonstrated in a dosage regime producing tolerable and rapidly reversible toxicity. The use of this drug in combination with other known active agents in the treatment of these diseases should be seriously considered.

REFERENCES

1. I. W. Kaplan, *Condylomata acuminata. New Orleans med. surg. J.* **94,** 388 (1942).
2. G. Falkson, A. G. Sandison and J. van Zyl, Podophyllin derivatives SPG 827 and SPI 77 in the treatment of advanced cancer. *S. Afr. J. Radiol.* **2,** 1 (1964).
3. O. Hubacher, Therapie karzinomatöser Endstadien mit SP-G und SP-I. *Deutsch Med Wschr.* **89,** 254 (1964).
4. O. Stamm, Zur Krebstherapie mit Podophyllin-Derivaten. Vorläufige Erfahrungen bei der zytostatischen Behandlung maligner Genitaltumoren mit SP-I und SP-G Sandoz. *München med Wschr.* **106,** 41 (1964).
5. V. K. Vaitkevicius and M. L. Reed, Clinical studies with podophyllum compounds SPI-77 (NSC 72274) and SPG-827 (NSC 42076). *Cancer chemother. Rep.* **50,** 565 (1966).
6. H. Stähelin, 4'-Demethyl-epipodophyllotoxin thenylidene glucoside (VM-26), podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6,** 303 (1970).
7. P. R. Gradwohl and H. Stähelin, Effect of VM-26, a podophyllotoxin glucoside derivative, on the uptake of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine by P-815 mas ocy oma cells *in vitro. Int. Cancer Congr. Abstr.* 10*th Congr., Houston, Texas,* p. 398 (1970).
8. F. M. Muggia, O. S. Selawry and H. H. Hansen, Clinical studies with a new podophyllotoxin derivative, epipodophyllotoxin, 4'-demethyl-9-(4,6-o-2-thenylidene-beta-D-glucopyranoside) (NSC-122819). *Cancer chemother Rep.* **55,** 575 (1971).
9. P. Dombernowsky, N. I. Nissen and V. Larsen, Clinical investigation of a new podophyllum derivative, epipodophyllotoxin, 4'-demethyl-9-(4,6-o-2-thenylidene-beta-D-glucopyranoside) (NSC-122819), in patients with malignant lymphomas and solid tumors. *Cancer chemother Rep.* **56,** 71 (1972).
10. G. Trempe, M. Sykes, C. Young, I. Krakoff, Phase I trial of the podophyllotoxin derivative VM 26. *Proc. Amer. Ass. Cancer Res.* **11,** 79 (1970).
11. European Organization for Research on the Treatment of Cancer, Clinical screening of epipodophyllotoxin—VM 26 in malignant lymphomas and solid tumors. *Brit. med. J.* **2,** 744 (1972).

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The Relative Biological Effectiveness of Cyclotron Fast Neutrons for Early and Late Damage to the Small Intestine of the Mouse

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Abstract—*The RBE for damage to the small intestine should be determined prior to treatment of cancer in the abdomen and pelvis with fast neutrons. It is particularly important to make certain that the RBE for late damage is no greater than the RBE for acute injury to this organ.*

In the present study the $LD_{50/5\ day}$ *following whole-body irradiation was used to measure the sentivity of the intestinal tract to acute injury. The* $LD_{50/90\ day}$, *following irradiation of a 4 cm segment of small intestine, was used to assess late damage to this structure. Relative to X-radiation, the fast neutron RBE for late damage* (1·9) *did not differ significantly from the fast neutron RBE for acute injury* (1·8). *The mechanism of death following partial intestine irradiation with neutrons or X-radiation was associated with bowel obstruction. A neutron dose of* 1·2 *krad delivered to only a small segment of the small intestine produced* 50% *mortality within* 90 *days.*

INTRODUCTION

THE RELATIVE biological effectiveness (RBE) for damage to the small intestine, a normal tissue at risk, should be determined prior to treatment of cancer in the abdomen and pelvis with fast neutrons. It is particularly important to make certain that the RBE for late damage is no greater than the RBE for acute injury to this organ. Several investigators have estimated the acute RBE for fast neutron damage to the intestine by measuring the $LD_{50/5\ day}$ or the survival of stem cells in the intestinal mucosa following whole body exposure [1–3]. The values obtained varied between 1·4 and 3·0, depending on the energy of the neutron beam used. However, in radiation therapy usually only a portion of the intestine is included in the treatment field. The potential late complications resulting from irradiation of only a segment of intestine include local mucosal ulceration, fibrosis, and stricture leading to luminal obstruction and death if not surgically corrected.

The purpose of the present study was to determine the RBE for fast neutron-produced late death of mice as a result of segmental small intestine exposure. $LD_{50/5\ day}$ measurements following whole-body irradiation also were made in order to compare in the same laboratory the RBE of fast neutrons for acute intestinal injury.

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MATERIAL AND METHODS

Animals

Female Swiss ICR mice (18–22 g) were obtained from Charles River Laboratory (Wilmington, Mass.) and were housed in the University of Washington vivarium for at least one week prior to experimentation. The animals were given Purina chow and water *ad libitum*.

Whole-body irradiation

Mice were retained in plexiglass boxes of sufficient size to allow them to turn around during irradiation and groups of 8 mice were exposed to graded doses of neutrons or X-rays. X-rays were generated by a General Electric Maxitron therapy unit (dose rate about 100 rad/min; 50 cm tube target to animal distance; 250 kVp; 0·5 mm Cu, 1 mm Al added filtration). The absorbed dose was measured with thermoluminescent dosimeters (TLD) surgically inserted in the abdomen of the animals. Neutrons of 8 MeV mean energy were produced by bombarding a beryllium target with 21 MeV deuterons at the University of Washington cyclotron. The animals were irradiated at 125 cm from the Be target at a neutron dose rate of about 35 rad/min. Neutron dosimetry was carried out with a tissue equivalent ionization chamber in conjunction with aluminum pellet activation analysis. Aluminum pellet activation was used to determine the dose rate in various areas of the beam.

Partial intestine irradiation

Under nembutal anesthesia, 4 cm segments of surgically exteriorized small intestines immediately proximal to the ceca were exposed to graded doses of X-rays or neutrons according to a modification of the procedure described by Osborne [4]. For X-irradiation, 4 animals were positioned around the sides of an 8×8 cm square aperture in a $\frac{1}{4}$ in. thick sheet of lead with the 4 cm segment of intestine located in the primary beam. For neutron irradiation a similar technique was employed. A square field was produced by means of a square aperture in an aluminum–bronze alloy, borated plastic collimator (Fig. 1). The radiation field produced by this collimator has been described previously [5]. To keep the intestine moist and to supply radiation dose "build-up" material, the exteriorized segment of intestine was sandwiched between several thicknesses of saline soaked gauze. The X-ray dose rate at 30 cm from the X-ray tube target was about 200 rad/min as determined with TLD. The neutron dose rate was about 175 rad/min at the intestine position as determined with a tissue equivalent ionization chamber. The segment of intestine was located 70 cm from the beryllium target.

Statistics

LD_{50} and RBE and their 95% confidence intervals were calculated by graphical probit analysis according to the procedure described by Litchfield and Wilcoxon [6].

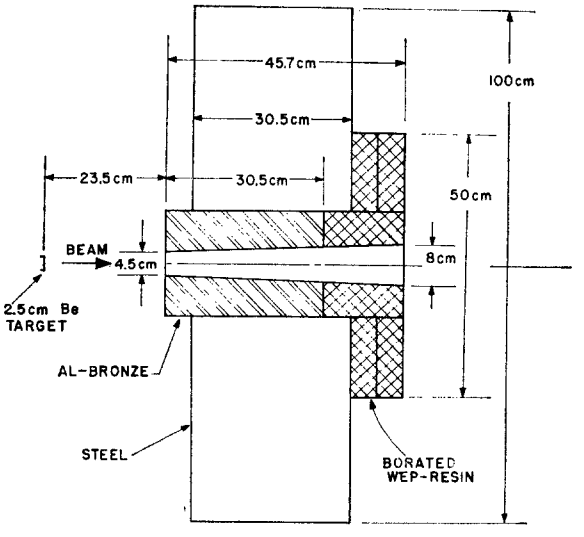


DIAGRAM OF RADIOBIOLOGY COLLIMATOR

Fig. 1. Biology collimator used for partial intestine irradiation with fast neutrons. Four anesthetized mice were positioned at the face of the collimator such that a 4 cm segment of the small intestine of each mouse protruded into the neutron beam while the rest of the animal was shielded.

RESULTS

The mortality curves used to estimate the $LD_{50/5\ day}$ for single whole-body neutron and X-ray exposures are shown in Fig. 2. The $LD_{50/5\ day}$ for X-radiation was 1·06 krad and for neutrons it was 0·59 krad, giving an RBE of 1·8.

Figure 3 illustrates the cumulative mortality after partial intestine irradiation with single exposures of neutrons or X-rays. The data show a high incidence of mortality during the second week after exposure followed by a slower rate of death. Most of the deaths observed occurred by 60 days but there were a few deaths as late as 9 months after irradiation. The data in Fig. 3 also indicate that when the ratio of mean X-ray dose to mean neutron dose was approximately 2:1, there was no difference in the cumulative mortality produced by the two types of radiation.

Figure 4 illustrates the gross pathology of the gastrointestinal tract observed at, or shortly before, death as a result of partial intestine irradiation with neutrons or X-rays. The animals exhibited distension of the small intestine proximal to bowel obstruction at the irradiated site. This response was seen in all animals examined that died between 6 days and 9 months post-irradiation. Post-mortem histological examination of the irradiated segment of bowel showed the lumen to be obliterated by inflammatory tissue in animals which

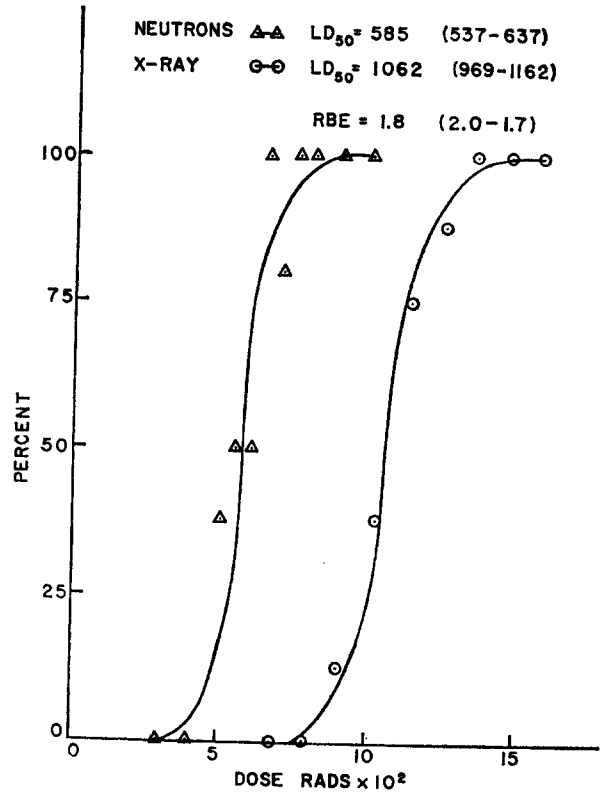


Fig. 2. Dose–mortality response 5 days following single whole-body neutron or X-ray exposures. Each point represents the per cent mortality of 7–8 animals. Best fit curves were determined by chi square analysis of probit curves. LD_{50} and RBE values are followed by the 95% confidence limits.

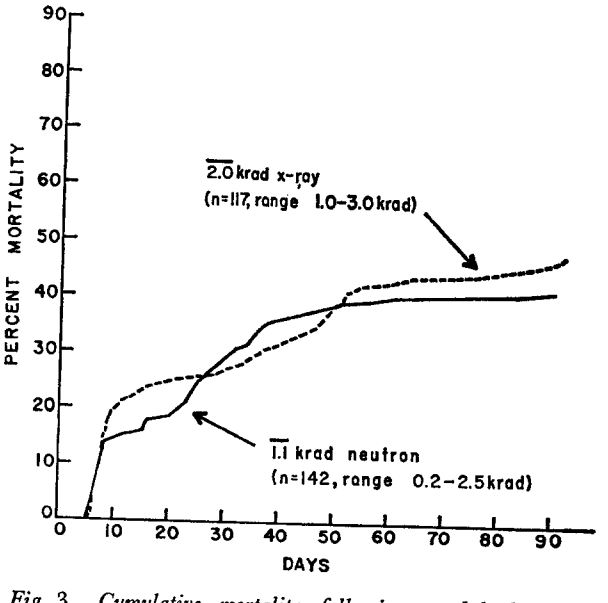


Fig. 3. Cumulative mortality following graded doses of neutrons or X-rays delivered to a 4 cm segment of small intestine.

succumbed during the second week (Fig. 5b). Animals dying after the second week had a reduced bowel lumen, hypertrophy of the muscularis, and sometimes a regenerated mucosa with blunted villi (Fig. 5c) as compared to the villi in a shielded area of the intestine (Fig. 5a). Adjacent to the irradiated segment the intestine was altered by vascular engorgement with focal hemorrhage, edema, and sparse and short villi (Fig. 5d). No unique differences were noted between X-irradiated and neutron-irradiated intestines.

The dose–mortality curves used to estimate the $LD_{50/90\ day}$ for partial intestine exposure are shown in Fig. 6. With X-rays the $LD_{50/90\ day}$ was 2·26 krad and with neutrons it was 1·18 krad, yielding an RBE of 1·9.

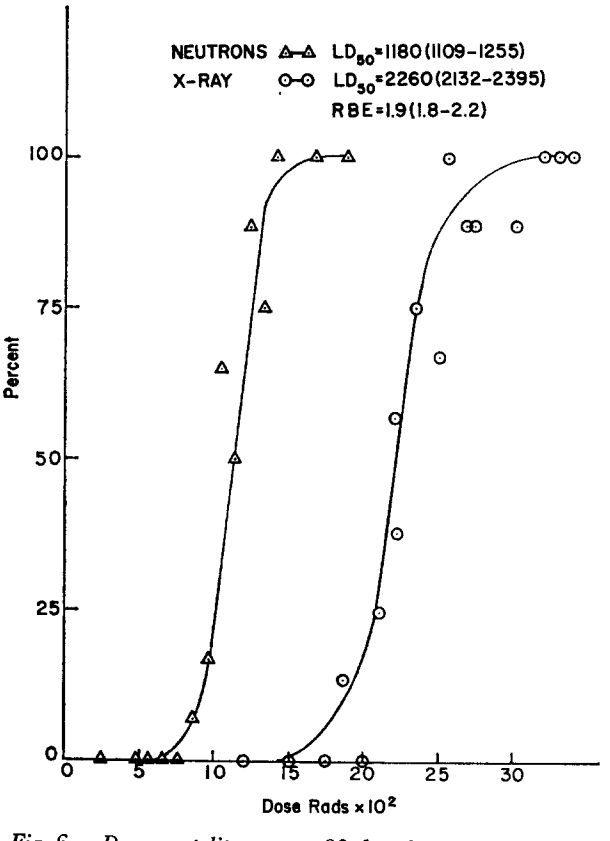


Fig. 6. Dose–mortality response 90 days following segmental intestine irradiation with neutrons or X-rays. Each point represents the per cent mortality of 8–12 animals. Best fit curves were determined by chi square analysis of probit curves. LD_{50} and RBE values are followed by the 95% confidence limits.

DISCUSSION

Animals receiving whole-body radiation exposures which result in gastro-intestinal death succumb within 5 days because of fluid and electrolyte loss, toxemia, and nutritional impairment [7]. In contrast, most of the animals receiving segmental intestine irradiation with fast neutrons or X-rays die from bowel obstruction between 6 and about 60 days after exposure. The high mortality rate which occurs

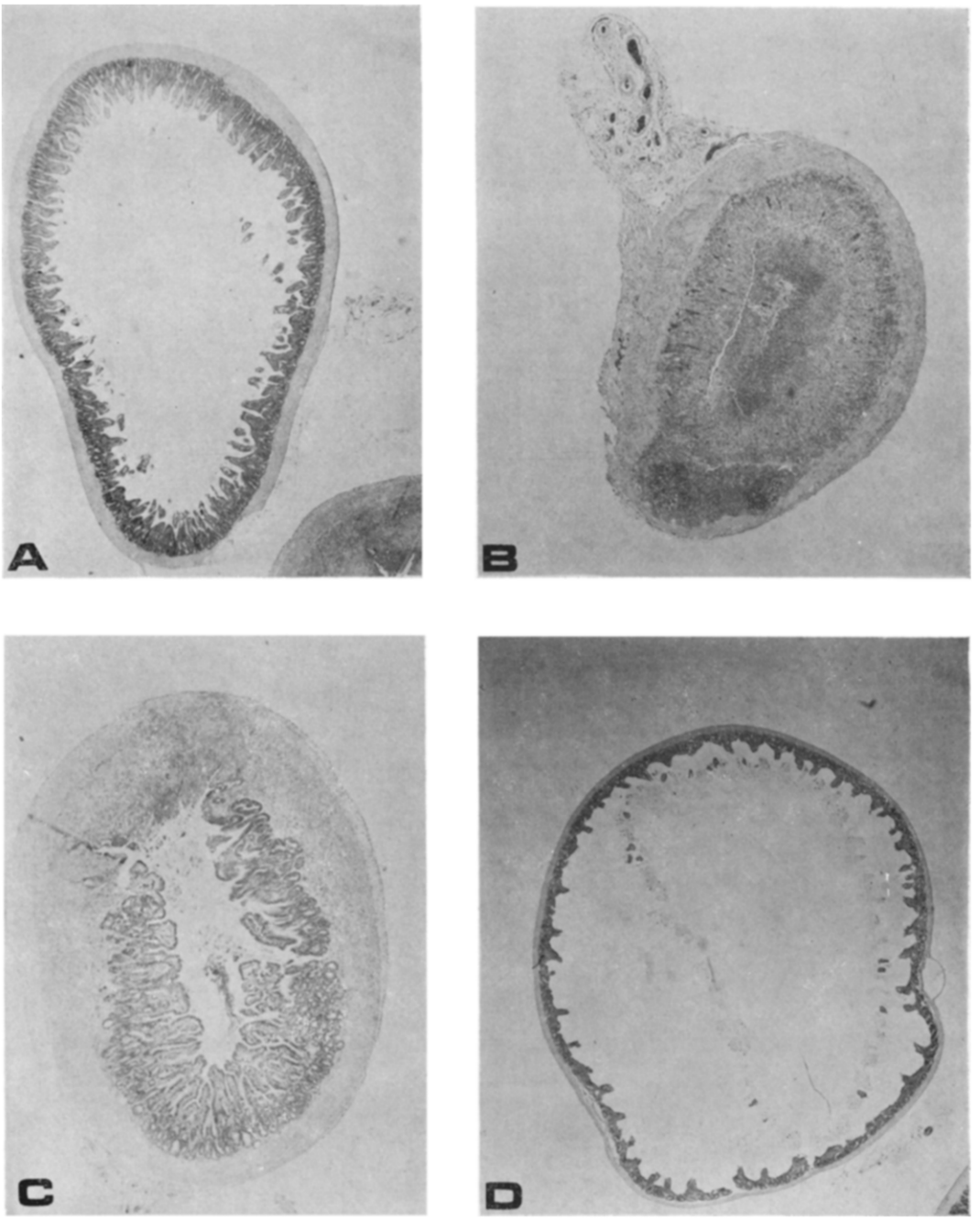


Fig. 5. *Histological cross sections of hematoxylin-eosin stained, shielded and irradiated small intestine.* (A) *Shielded area of intestine from an animal which died* 25 *days after segmental intestinal exposure.* (B) *Segment of irradiated intestine of an animal which died* 12 *days after exposure.* (C) *Segment of irradiated intestine of animal which died* 34 *days after exposure.* (D) *Segment of intestine adjacent to a bowel obstruction of animal which died* 25 *days after exposure.*

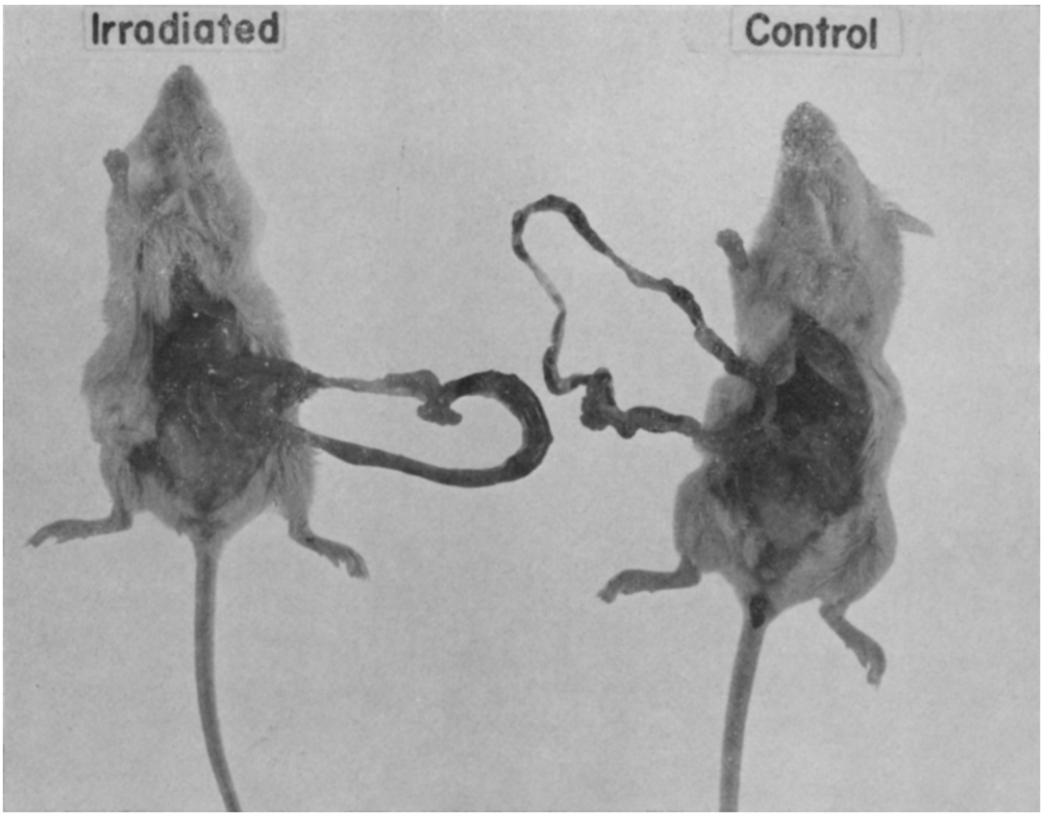


Fig. 4. *Gross pathology resulting from neutron or X-ray exposure of a* 4 *cm segment of small intestine.*

[to face p. 102

during the second week (Fig. 3) post-exposure appears to result from complete obliteration of the lumen by inflammatory tissue (Fig. 5b) whereas later deaths are associated with stricture (Fig. 5c). However, the potency of neutrons relative to X-radiation for production of these forms of late damage to the intestine is not significantly different from that for production of acute intestinal damage. The confidence limits of the RBE for the $LD_{50/5\ day}$ overlapped those of the RBE for the $LD_{50/90\ day}$ (Fig. 2 and 6). Thus, the intestine is similar to skin [8] in that there is little difference in the neutron RBE values for early *v* late responses in these tissues.

The neutron $LD_{50/90\ day}$ of 1·18 krad for late death from irradiation of only a small segment of the intestine may be of clinical importance. In the treatment of intrapelvic and intra-abdominal cancers by protracted, fractionated neutron exposures the accumulated dose to portions of the small intestine may exceed 1·2 krad. This becomes important when one considers that the rather large capacity of the small intestine to repair injury following photon irradiation is markedly reduced with fast neutrons [3, 9–11]. Therefore, the possibility of intestinal damage should be seriously considered in fast neutron therapy of abdominal and pelvic tumors where the same segment of intestine is repeatedly exposed.

Acknowledgments—Appreciation is expressed to the Medical Radiation Physics Division for the X-radiation and neutron dosimetry. This work was supported by U.S. Public Health Service Grant CA 12441 and Research Career Development Award CA 41735.

REFERENCES

1. 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).
2. J. J. Broerse, G. W. Barendsen, G. Freriks and L. M. Van Putten, RBE values of 15 MeV neutrons for effects on normal tissue. *Europ. J. Cancer* **7,** 171 (1971).
3. S. Hornsey, S. Vatistas, D. K. Bewley and C. J. Parnell, The effects of fractionation on four-day survival of mice after whole-body neutron irradiation. *Brit. J. Radiol.* **38,** 878 (1965).
4. J. W. Osborne, K. N. Prasad and G. R. Zimmerman, Changes in the rat intestine after X-irradiation of exteriorized short segments of ileum. *Radiat. Res.* **43,** 131 (1970).
5. P. Wootton, H. Bichsel, J. Eenmaa, R. G. Parker, K. Weaver, D. L. Williams and W. G. Wyckoff, The University of Washington Fast Neutron Beam Therapy Facility. *Boston Workshop on Practical Clinical Criteria for a Fast Neutron Generator,* May 28–29, (1973).
6. J. T. Litchfield and F. W. Wilcoxon, A Simplified method of evaluating dose-effects experiments. *J. Pharmacol. expt. Therapy* **96,** 99 (1949).
7. V. P. Bond, T. M. Fliedner and J. O. Archambeau, *Mammalian Radiation Lethality.* Academic Press, New York (1965).
8. S. B. Field, Early and late reactions in skin of rats following irradiation with X-rays or fast neutrons. *Radiol.* **92,** 381–384 (1969).
9. K. Nakagawa, Intestinal damage following a single and fractionated irradiation with fast neutrons. *Nippon Igaku Hoshasen Gakki Zasshi* **29,** 241–450 (1969). (In *Nucl. Sci. Abstr.* **24,** 290, 1970).
10. J. J. Broerse and H. Roelse, Survival of intestinal crypt cells after fractionated exposure to X-rays and 15 MeV neutrons. *Int. J. Radiat. Biol.* **20,** 391–395 (1971).
11. 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,** 796–801 (1970).

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Cell Reactivity Towards Syngeneic Neoplastic Cells in Mice Hypersensitized to Dinitrophenol*

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Abstract—*Three groups of 12-week old inbred Balb/c mice were sensitized by 3 weekly skin paintings with 1-fluoro-2,4-dinitrobenzene. One week later, one group and a further unsensitized group were inoculated with a non-inducing dose of adenocarcinoma (ADK-lt) cells coated with dinitrophenol, one group received an identical dose of uncoated tumor cells and the third sensitized group was not treated. After 15 days, these four groups and a further untreated group were challenged with 5×10^5 ADK-lt cells. During the following three weeks, the presensitized and prime group displayed delayed tumor appearance and slower tumor growth. Extensive necrosis and massive parvicellular infiltration were noted in 80% of tumors from animals in this group.*

INTRODUCTION

MOST HUMAN and animal tumors contain tumor-specific transplantation antigens (TSTA) exposed on the cell membrane. Some of these are peculiar to individual tumors whereas others are common to tumors of different types. In most cases, they are poorly immunogenic in the autochthonous host or syngeneic animals.

This may be the result of genetic tolerance towards these membrane antigens on the part of bearer animals [1]. Alternatively, TSTA's may only differ from membrane antigens in one or a few determinants, in the sense that carrier determinants are missing [2].

Attempts have been made to increase neoplastic cell immunogenicity in syngeneic hosts by coupling several antigen determinants with membrane proteins [2–4]. These have not led to significant changes in bearer animal resistance to syngeneic tumors, probably because the reactivity thus induced is humoral, rather than cellular. Resistance to tumors is, in fact, cell-mediated and some antibody classes may interfere with cell reaction mechanisms [5].

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The present paper reports an attempt to obtain specific cell reactivity to TSTA in mice by induction of cell-mediated immunity by skin painting with a hapten, followed by challenge with live neoplastic cells coupled with the same hapten. Resistance towards high cell doses was not significantly influenced, though a marked increase in cellular reactivity was noted.

MATERIALS AND METHODS

Mice

Five groups of twenty 12-week-old, 20–22g Balb/c mice, maintained by continuous brother–sister mating and originally obtained from the National Institute of Health (U.S.A.) were used.

Tumor

A mammary adenocarcinoma (ADK-lt) that arose spontaneously within our colony and was maintained for 12 generations was transmitted by subcutaneous inoculation of various numbers of teased cells in 0·5 ml of an Eagle–MEM suspension. Cell viability was determined by Trypan blue dye exclusion according to Takahashi *et al.* [6]. The injection site was palpated for presence of tumor every two days and growth rates measured with calipers [7].

Dinitrophenolisation of neoplastic cells

Tumor cell suspensions were centrifuged for 10 min at 4°C, 1 ml of packed cells was diluted in 4 ml Earle (pH 8·5) containing 10 mg 2,4-dinitrobenzene sulphonic acid sodium salt (Eastman Kodak, Rochester, New York) per ml and gently stirred for 30 min at 37°C. Two washings with Earle containing 10% calf serum were followed by 15 min gentle stirring at room temperature. After a final washing, cells were brought to the required concentration. Cell viability fell about 12% during dinitrophenolisation. Surface labelling with dinitrophenyl (DNP) groups was tested by agglutination with an anti-DNP_{26}–BSA rabbit antiserum on microplates, using 200 μl of cell suspension (5×10^7/ml). After 3 hr incubation at 37°C a marked agglutination was noted down, with coated cells only, to a serum dilution of 1 : 128. At this point, it was specifically inhibited by preincubation with 5×10^4 mM of DNP–lysine (Koch-Light Lab., Colnbrook, England). Direct quantitative determition of DNP groups bound to the cell membranes was not attempted, attention being confined to the establishment of membrane integrity and the proliferative capacity of the incubated cells. Cell viability is, in fact, an essential requisite for antigenicity [3, 8] and is also inversely proportional to the number of DNP groups bound to the cell wall [9].

Skin painting

Skin painting, as described by Iverson [10] was carried out with 1-fluoro-2,4-dinitrobenzene (FDNB) (K & K Lab., Plainview, Cal).

Histological examination

Thirty days after challenge, all four groups were killed. Tumors were excised, fixed in Serra, embedded in parafin, sectioned and stained with haematoxylin–eosin. The mean volume of the necrotic area and the extent of the parvicellular infiltrate were evaluated on photographic reconstructions of the serial sections.

RESULTS

Viability and activity of ADK-lt cells coated with DNP

Trypan blue exclusion showed that 80% of the tumor cells remained viable after coating. Their ability to induce neoplasia in the host was assessed by subcutaneous inoculation of scalar doses. A control was run on mice inoculated with non-coated cells. The number of viable, coated cells required to induce neoplasia was double that of uncoated cells. This was due depressed proliferative capacity on the part of coated cells (Forni, unpublished data).

The effect of presensitizing to DNP and priming with DNP-coated cells

Three goups of animals (A, B and C) were painted with FDNB once a week for 3 weeks to ensure presensitisation to DNP. One week later, group A and an untreated group (D) were inoculated subcutaneously on the left side with a non inducing dose (1×10^3) of viable DNP-coated cells. Group C received an identical dose of untreated tumor cells. After a further two weeks, groups A, B, C and D and a second hitherto untreated group (E) were challenged with 5×10^5 viable ADK-lt cells, subcutaneously injected in the right groin. This dose is sufficient to induce a tumor incidence of 90% in mice of this age [7]. Tumor growth rates were followed until sacrifice after a further 30 days.

Delay tumor onset was noted in group A during the first three weeks. After the 25th day, however, incidences were the same in all 4 groups (Fig. 1). The same picture was observed with respect to tumor growth: as can be seen in Fig. 2 only starting from the 20th day the neoplastic masses of group A grew at the same rate as the other groups.

FDNB-painted mice (groups A, B and C) displayed a marked increase in circulating leukocytes. Spleen enlargement was noteworthy in group D only (Table 1).

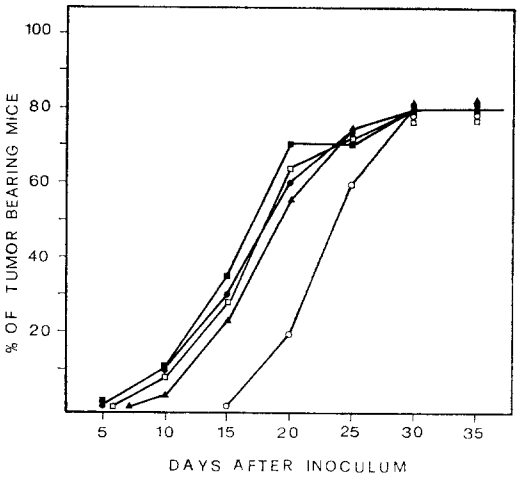


Fig. 1. Incidence of tumors after challenge with 5×10^5 ADK-lt cells.

(○): group A, mice skin painted and primed with DNP-coated ADK-lt cells; (■): group B, skin painted only; (□): group C, skin painted and primed with uncoated cells; (▲): group D, not sensitized and primed with DNP-coated ADK-lt cells; (●): group E, controls.

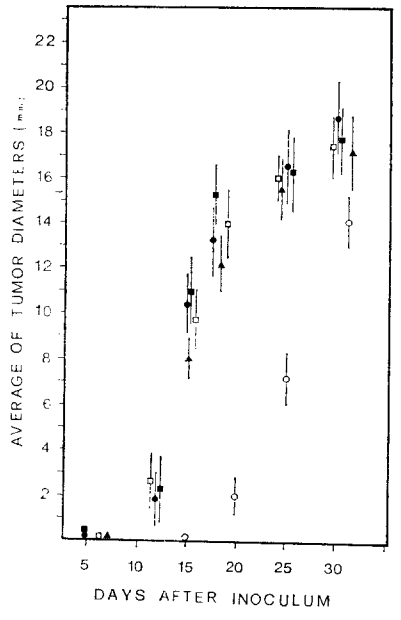


Fig. 2. Tumor growth rates after challenge with 5×10^5 *ADK-lt cells.*

(○): group A, mice skin painted and primed with DNP-coated ADK-lt cells; (■): group B, skin painted only; (□): group C, skin painted and primed with uncoated cells; (▲): group D, not sensitized and primed with DNP-coated ADK-lt cells; (●): group E, controls.

DISCUSSION

Our data show that increased cell reactivity towards TSTA's could be achieved by presensitisation with DNP and priming with a non-inducing dose of live tumor cells coated with the same hapten. The cell reaction so induced delayed tumor appearance and induced massive leukocyte infiltration. In fact, extensive necrosis was noted in sensitised and primed animals thirty days after challenge with a tumor-inducing dose. When presensitisation or priming was carried out alone or with uncoated cells, however, no change in cellular reactivity occurred.

These results do not agree with those obtained by Martin *et al.* [11]. Using spleen cells from mice immunised with syngeneic tumor cells coated with 2,4-diphenylaminocaproate (DnpC), these workers noted that, while increased reactivity in vitro was indeed obtainable, prior skin painting with FDNB actually brought about a decrease. This marked contrast with our findings may be attributable to differences in coating method and the use of different evaluation parameters.

Table 1. Circulating white blood cell counts and spleen weights in the four groups of mice at 30 days tumor cell challenge

	White blood count (cells/mm^3)	Spleen weight (mg)
Group A (skin painted and primed with ADK-lt coated cells).	8,000 ± 750	190 ± 30
Group B (skin painted only).	11,200 ± 850	200 ± 25
Group C (skin painted and primed with uncoated ADK-lt cells).	11,700 ± 700	190 ± 30
Group D (not sensitised and primed with ADK-lt coated cells).	5,500 ± 600	310 ± 40
Group E (controls).	5,800 ± 650	180 ± 20

Ninety per cent of group A tumors presented extensive areas of eosinophile necrosis with pronounced pycknosis. Serial sections showed necrosis from 60 to 80% to the entire tumor mass. Massive parvicellular infiltration was observed both around the areas of necrosis and throughout the tumor. Necrosis extending for more than 6% of the neoplastic mass, on other hand, was noted in only one tumor in group B and another in group E. In this case, cellular infiltration was peripheral only. Neither necrosis nor infiltration were present in group C and D tumors.

Our experiment was conducted *in vivo* with a neoplastic cell dose giving 90% tumor induction. No lasting reduction in tumor incidence was achieved in presensitised and primed animals under these conditions. To this extent, our findings agree with those obtained with a similar system *in vivo* by Bauminger and Yachnin [8].

Preimmunisation with DNP-treated cells, combined with induction of hypersensitivity to

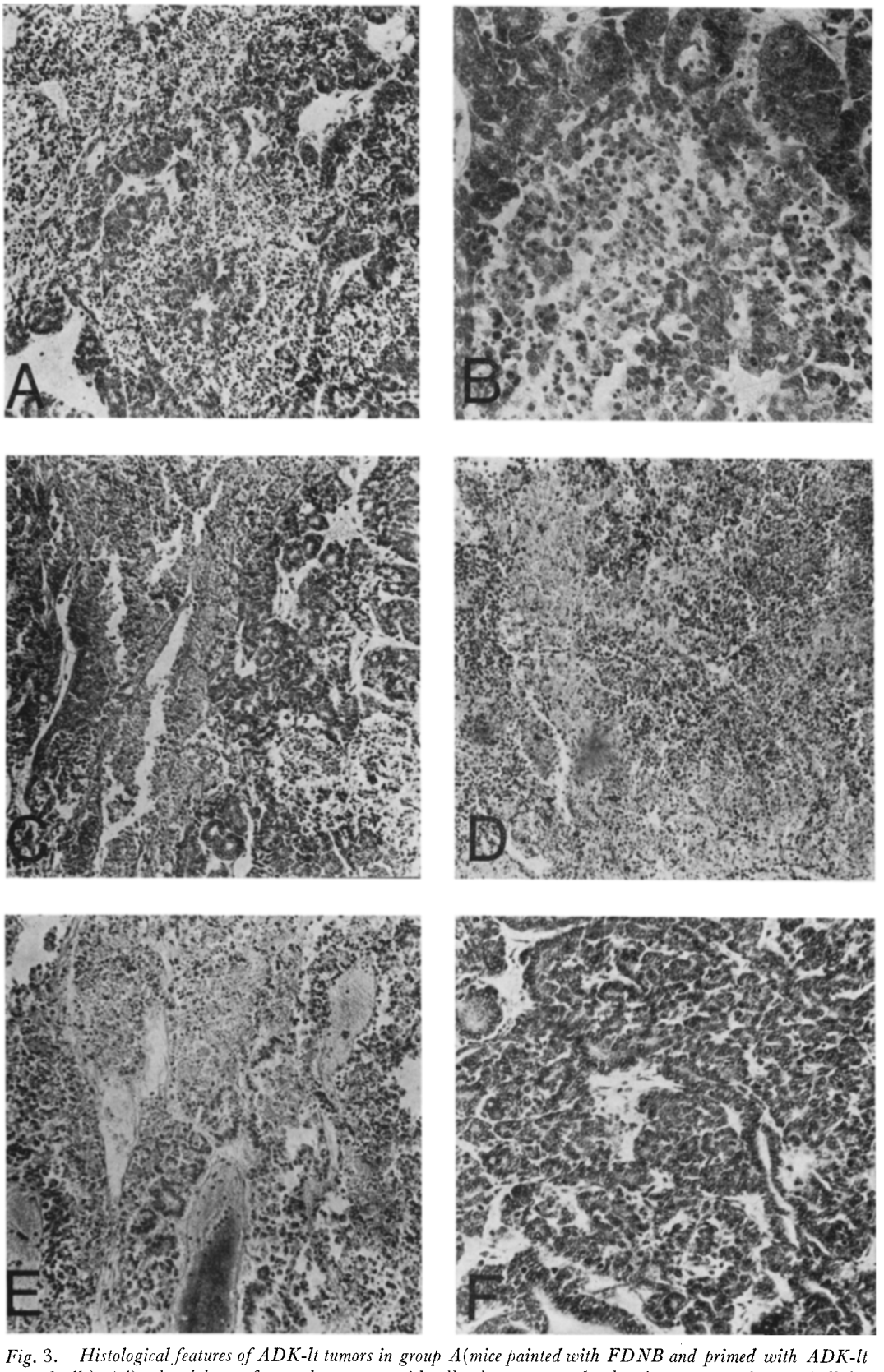


Fig. 3. *Histological features of ADK-lt tumors in group A(mice painted with FDNB and primed with ADK-lt coated cells). (A): breakdown of normal structure, with cell enlargement and pyknosis; note massive parvicellular infiltration of the neoplastic mass,* 50 ×. *(B): same section at* 500 ×. *(C): homogeneous areas of necrosis associated with the previous picture of degeneration,* 50 ×. *(D, E): complete structural alteration with massive parvicellular infiltration,* 50 ×. *(F): controls. No evidence of necrosis and infiltration,* 12 ×.

[to face p. 106]

DNP, caused an initial reduction in tumor growth rate and resulted in the appearance of marked cell infiltration. This suggests that DNP groups bound to the tumor cell membrane may act as suitable carriers for antigens only in cases where the formation of helper cells [12] specifically reactive for DNP groups has been induced.

REFERENCES

1. B. Benacerraf and McDevitt, Histocompatibility-linked immune response genes. *Science*, **175,** 273 (1972).
2. N. A. Mitchison, Immunologic approach to cancer. *Transplant. Proc.* **2,** 92 (1970).
3. O. J. Plescia and W. Braun, Control of neoplasia by immunological means: an assessment of a new approach. *G. Batt. Virol.* **63,** 7 (1970).
4. W. Braun, O. J. Plescia, J. Raskova and D. Webb, Basic proteins and synthetic polynucleotides as modifiers of immunogenicity of syngeneic tumor cells. In *Immunological Parameters of Host–Tumor Relationships*, edited by D. H. Weiss, Academic Press, New York (1971).
5. I. Hellstrom and K. E. Hellstrom, Cellular immunity and blocking antibodies to tumors. *J. reticuloendoth. Soc.* **10,** 131 (1971).
6. T. Takahashi, L. J. Old and E. A. Boyse, Surface antigens of plasma cells. *J. exp. Med.* **131,** 1325 (1970).
7. G. Forni and P. M. Comoglio, Growth of syngeneic tumors in unimmunized newborn and adult hosts. *Brit. J. Cancer* **27,** 120 (1973).
8. S. Bauminger and S. Yachnin, Unimpeded growth of tumor in host pre-immunized with tyrosyl or dinitrophenyl coated tumor cells. *Brit. J. Cancer* **26,** 77 (1971).
9. H. Wagner, Cell-mediated immune response *in vivo*. IV. Metabolic studies on cellular immunogenicity. *Eur. J. Immunol.* **3,** 84 (1973).
10. G. M. Iverson, Ability of CBA mice to produce anti-idiotipyc sera to 5563 myeloma protein. *Nature (Lond.)* **227,** 274 (1970).
11. W. H. Martin, J. Wunderlich, F. Flecher and J. K. Inman, Enhanced immunogenicity of chemically coated syngeneic tumor cells. *Proc. nat. Acad. Sci.* **68,** 469 (1971).
12. R. B. Taylor and G. M. Iverson, Hapten competition and the nature of cell-cooperation in the antibody response. *Proc. roy. Soc. Lond. B.* **176,** 393 (1971).

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Early Biochemical Changes in Phytohemagglutinin-Stimulated Peripheral Blood Lymphocytes from Normal and Tumor–Bearing Rats

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Abstract—*The very early increase in the intracellular concentration of 3′,5′-cyclic GMP and in the rate of incorporation of ^{33}P into membrane phospholipid, and the early increases in the level of uridine kinase and cystathionine synthetase upon stimulation of peripheral lymphocytes with phytohemagglutinin, correlate well with the standard assay for lymphoblastic transformation. The rapidity. lower variability and in particular greater sensitivity of assays based on earlier events, suggests that they may be useful adjuncts for assessing immunocompetence under specified conditions, and in combination, may aid in diagnosing the molecular lesion when immunity is impaired. Coincidentally this study also shows that the peripheral blood lymphocytes from animals bearing transplantable tumors are inherently incompetent, and also that the serum from such animals suppresses the transformation of lymphocytes from normal animals. The immunosuppressive effect of the transplantable tumors was independent of the adrenal cortex.*

INTRODUCTION

MANY of the molecular events which occur during the blastic transformation of peripheral blood lymphocytes in response to phytohemagglutinin (PHA) stimulation [1–8] bear a striking resemblance to those observed in the regenerating liver following partial hepatectomy [9–12]. It is now generally accepted [13] that PHA is relatively specific for stimulation of thymus-dependent lymphocytes, i.e. the fraction of the lymphocytes which are involved in cell mediated immunity. The standard assay for blastic transformation, based on the increased rate of DNA synthesis as measured by tritiated thymidine incorporation [2], involves the culturing of lymphocytes in the presence of a specific or nonspecific mitogen for three to seven days with concomittant or subsequent labelling of the DNA with tritiated thymidine. Although the sensitivity of the latter assay can be increased to acceptable levels by increased labelling time, there is a marked variability (over 4-fold) in the extent of thymidine incorporation into DNA by lymphocytes from different individuals of presumably normal immunocompetence [14], and it also requires that serum be present in the culture medium. Recent studies on the early biochemical events associated with lymphoblastic transformation have coincidentally provided other potential assays for lymphocyte transformation, which in some cases are of equal or greater sensitivity, have less variability, and in all cases are more rapid that the present assays based on increased DNA synthesis. It was the purpose of the present study to determine the feasibility of using one of these assays

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as an alternative *in vitro* measure of the cell mediated immune response where rapidity or sensitivity are important.

The importance of cellular immunity in surveillance, remission and other host-tumor relations in animals and man has been emphasized in several recent studies [15–17]. It seems clear that the answers to such problems as the relationship between the development of malignancy to defects in the immune system the prognostic implications of cutaneous anergy, and the immunological status of the cancer patient throughout therapy, including the responsiveness of the lymphocytes to specific tumor antigens, would be greatly facilitated by a more practical and sensitive *in vitro* assay which measures the potential for lymphoblastic transformation. The assay should be practical in view of the large number of assays required to establish immunological profiles and sufficiently sensitive to detect the stimulation of only a small percentage of the total lymphocytes by a specific tumor antigen. The results of the present study suggest that the measurement of the intracellular concentration of cyclic GMP may satisfy these requirements. The use of this assay and others involving early biochemical events in lymphoblastic transformation should also aid in pinpointing the site of the molecular lesion in those cases in which immunity has been shown to be impaired.

MATERIAL AND METHODS

(*a*) *Lymphocyte preparation*

Peripheral blood, obtained from normal or tumor bearing rats of the Sprague–Dawley or Buffalo Strain by cardiac puncture, was drawn directly into a heparinized syringe (1000 units/ml of whole blood) and allowed to settle for 45 min at 37°. The upper lymphocyte rich layer was removed, centrifuged, washed once and the sedimented cells were taken up in the indicated medium (cf. below). An aliquot of the lymphocyte suspension was counted under a light microscope to determine the number of nucleated cells. This cell preparation, which contains platelets and granulocytes as well as lymphocytes, was used for all assays. The Buffalo strain rats bearing Hepatomas 5123D, 5123TC or 7800 [12] were shipped by H. P. M., Howard University, Washington to the Columbus Laboratory, while the mice bearing Sarcoma S-37 [18] were obtained from Dr. R. H. Matthews, Ohio State University, Columbus, Ohio. Where indicated the animals received 40 mg/kg body weight of dexamethasone phosphate (Sigma Chemical Co., St. Louis, Mo.) 18 hr before removal of the lymphocytes. Similarly, adrenalectomized rats were used 5–7 days postoperatively. Sera, prepared by centrifugation of a sample of peripheral blood for 15 min at $10{,}000 \times g$ to remove all cells, was tested in the *in vitro* assays either immediately after preparation, or after a single freezing.

(*b*) *Tritiated thymidine incorporation assay*

This assay [12] measures the rate of incorporation of tritiated thymidine (^{3}HTdr) into lymphocyte DNA. Lymphocytes (5×10^6) are suspended in 5 ml of Eagles medium containing 100 μg/ml of penicillin G. PHA-M was added to one half of the cultures at a concentration of 250 μg/ml and where indicated, dexamethasone was added to 1·0 mM. Following the addition of normal rat serum (5%, V/V) and the incubation of the cultures in air at 37° for 36hr, ^{3}HTdr was added to a concentration of 2μCi per ml and the incubation was continued for an additional 12 hr. The cultures were chilled and following sedimentation by low speed centrifugation, the cells were washed once with saline (0.9%), twice with 5% trichloroacetic acid and twice in 95% ethanol; the final pellet was dissolved in solubilizer for counting in liquid scintillant.

(*c*) *Cyclic GMP assay* [19]

Lymphocytes (10^6 per 0·5 ml) were suspended in Hanks balanced salt solution and PHA-M (Difco Laboratories, Detroit, Mich.), dexamethasone (1·0 mM) and serum were added where indicated. The final volume was adjusted to 0·5 ml. Lymphocytes were not preincubated prior to the experiment but were kept at 0° until all reaction components were added. The material precipitating in 10 min at 0° was removed by centrifugation and the supernatant was either (1) extracted with acid-saturated ether to remove the trichloroacetic acid, or (2) chromatographed on a column of Biorad AG-1-X8. For chromatography the lymphocyte extract was adjusted to pH 7·5 with sodium carbonate and applied to a 1 × 5 cm column of Biorad AG-1-X8 equilibrated with 0·05 N Na formate, pH 7·5. The column was washed with 5 ml of this same buffer, followed by 5 ml of 0·1N formic acid and 5 ml of 1·0N formic acid. The cyclic GMP subsequently eluted with 5 ml of 4·0N formic acid, was lyophylized, then taken up in 0·05 M sodium acetate buffer for radio-

immunoassay. The aqueous phase was neutralilized with bicarbonate prior to assay for cycic GMP using a commercially available radioimmunoassay kit (Collaborative Research Inc., Waltham, Mass). In this assay [cf 19] cyclic GMP in the unknown competes with ^{125}I-succinyl cyclic GMP for binding sites on a specific rabbit cyclic GMP antibody. After 2 hr at 0°, sheep anti-rabbit immunoglobulin G is added and allowed to precipitate. The amount of cyclic GMP in the unknown sample is read from a standard curve. Standard recovery tests were performed with the radioimmunoassay by adding known amounts of ^{3}H-cyclic GMP to the trichloroacetic acid extract of lymphocytes. In all cases duplicate or triplicate samples were carried through the entire assay procedure, i.e. from incubation to final cyclic GMP determination.

(*d*) *Cystathionine synthetase assay* [6]

Lymphocytes were cultured as for the ^{3}H-thymidine incorporation assay. After a 24 hr incubation the cells, removed from the culture fluid by centrifugation, were frozen, then suspended in 1·0 ml of buffer containing 30 mM Tris, pH 8·3, 1·3 mM pyridozal phosphate, then frozen and thawed three more times. Cell debris was removed by centrifugation and 50 λ of the supernatant was incubated for 4 hr at 37° with 50 λ of a solution containing 150 mM Tris pH 8·3, 2·5 mM EDTA, 0·65 mM pyridoxal phosphate, 0·17 mM cystathionine, 10 mM homocysteine and 6·60 nmoles of 3-^{14}C serine (300–330 x 10^3 counts/min). 50 λ of this incubation mixture was applied to a cellulose TLC plate (Eastman Kodak Co.) and developed overnight in a solvent system containing isopropanol: formic acid: H_2O (80:6:20). The area corresponding to cystathionine synthetase was scraped off and counted.

(*e*) *Uridine kinase assay* [8, 11]

Lymphocytes were cultured as for ^{3}H-thymidine incorporation. After 24 hr the cells were removed from culture by centrifugation, washed once in Hanks balanced salt solution, and frozen until assayed. The cell pellet was thawed and resuspended in 0·2 ml of 20 mM Tris HCl, pH 7·5, 10 mM $MgCl_2$, and then refrozen and thawed twice more. The cellular debris was removed by centrifugation and the supernatant assayed as previously described [11].

(*f*) *Phospholipid assay* [3]

^{33}Phosphorus incorporation into membrane phospholipid was assayed according to the procedure of Fisher and Mueller [3]. Lymphocytes were incubated in 0·5 ml of phosphate-free Hanks balanced salt solution containing PHA-M and dexamethazone as indicated. 50 μCi of Na_3 $^{33}PO_4$ in 50 μl (I.C.N., Cleveland, Ohio) was added to each tube before incubation at 37°C for 30 min. The cells were removed by centrifugation, washed 2 times with 0·4 ml of 5% TCA, then extracted with 0·35 ml of 2:1 chloroform: methanol; 0·1 ml of H_2O was added and the chloroform layer was removed for spotting on a silica TLC plates (Eastman Kodak Co.). The latter were developed for 4 hr in $CHCl_3$: MeOH: H_2O (75: 25: 2) and the area corresponding to the solvent front was removed and counted.

RESULTS

An earlier study by Hadden *et al.* [5] suggested that one of the earliest biochemical events in PHA-stimulated human lymphocytes is an immediate rise in intracellular 3′,5′-cyclic GMP. This led to the postulate that the elevation of the concentration of this cyclic nucleotide may initiate events leading to cell division. Shown in Fig. 1 (a) is a plot of the intracellular

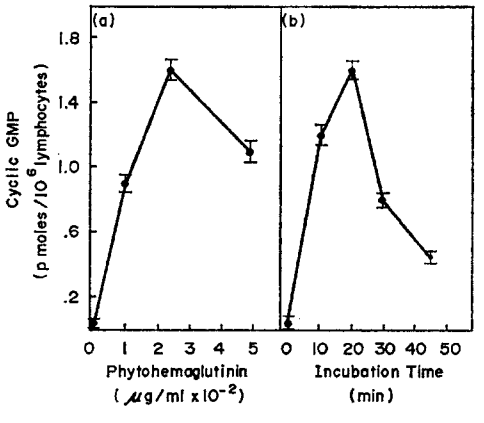


Fig. 1. Changes in the concentration of cyclic GMP in lymphocytes in response to stimulation with phytohemagglutinin. (a) The concentration of cyclic GMP in lymphocytes 20 min after stimulation with phytohemagglutinin, as a function of the concentration of the mitogen. (b) The concentration of cyclic GMP in lymphocytes as a function of time of incubation following addition of an optimal concentration of the mitogen. The values given represent the average of at least three experiments ± the standard deviation.

concentration of cyclic GMP in normal rat lymphocytes 20 min after incubation *in vitro* with phytohemaglutinin (PHA), as a function of the concentration of the added mitogen. The indicated optimal concentration of 250 μg/ml of PHA was selected for further studies.

Figure 1(b) shows the temporal change in the intracellular concentration of cyclic GMP in rat peripheral blood lymphocytes incubated in the presence of PHA. As in human lymphocytes [5], the intracellular concentration of cyclic GMP in the unstimulated lymphocytes is extremely low (0·05 pmoles/10^6 nucleated cells), but rises immediately upon stimulation to a transient maximum within approximately 20 min. In the case of lymphocytes from normal adult rats, this maximum was found to be consistently 70- to 80-fold higher than that of the unstimulated lymphocyte population. Recovery studies showed that 85% of the ^{3}H-cyclic GMP was recovered by both the acidified ether extraction method and by chromatography on Biorad AG-1X8. Treatment by cyclic nucleotide diesterase destroyed all of the ^{3}H-cyclic GMP as expected. Thus, the radioimmunoassay appears to be measuring cyclic GMP and not some other contaminating nucleotide; thus the changes detected by this assay upon stimulation of lymphocytes with PHA reflect changes in the intracellular concentration of cyclic GMP.

Several other early events in lymphoblastic transformation have been recently reported in the literature and represent other potential assays for immuno-competence. These events include an increase of ^{32}P incorporation into membrane phospholipid within 30 min of incubation in the presence of PHA [3], and after 24 hr of culture, an increase in the levels of uridine kinase [8] and a rise in cystathionine synthetase; the latter enzyme is practically undetectable in unstimulated lymphocytes [6]. All three of these events occur before the major increase in DNA synthesis which is seen at 3 days.

The changes in these parameters as well as in the cyclic GMP concentration were correlated with the tritiated thymidine incorporation into DNA in PHA-stimulated lymphocytes; the latter assay is the standard *in vitro* assay for the status of cellular immunity *in vivo*. Included in these studies were lymphocytes from normal rats, rats immunosuppressed by treatment with dexamethazone, and rats bearing transplantable hepatomas. The data in Table 1 indicate that there was a 16-fold stimulation of tritiated thymidine incorporation over a 12 hr period in a 48 hr culture of PHA stimulated normal lymphocytes, as compared to nonstimulated control cultures. The cyclic GMP concentration was increased 70- to 80-fold after only 20 min of incubation with the mitogen, the ^{33}P incorporation increased 7-fold within 30 min and, cystathionine synthetase and uridine kinase were increased 4·6- and 4·1-fold, respectively after 24 hr. The reliability of these assays is supported by the fact that the stimulation of the blastogenesis of lymphocytes by PHA as measured by all assays was drastically reduced when cells were derived from dexamethazone treated and thus immunosuppressed animals. Similar inhibition could be obtained by adding 1·0 mM dexamethazone directly to the incubation medium (results not shown).

Of special interest are the results of the incubation of lymphocytes from rats bearing the transplantable Hepatomas 5123D and 7800 which show that on the basis of all the events assayed, there is a drastic decrease in the PHA response. This abnormal response was present within two weeks after innoculation of the tumor cells into the hind legs of the rats and was maintained throughout the growth of the tumor. The immune response was increased only 20% in the tumor bearing rats after adrenalectomy indicating that the tumor and not high levels of circulating adrenocorticoids was primarily responsible for the immunosuppression.

The lymphocytes were not exposed to serum from tumor bearing rats in the experiment shown in Table 1 so that the defects observed were inherent in the lymphocytes themselves. However, as shown in Table 2, a factor(s) is present in the serum of tumor bearing, but not normal animals, which inhibits the blastic transformation of normal lymphocytes in response to PHA. Using the cyclic GMP assay, the cystathionine synthetase assay or tritiated thymidine incorporation assay, with normal serum present the stimulation was as high as in the absence of serum. This as yet unidentified factor(s) is present early in the tumor growth and found in both rats bearing the transplantable hepatomas and in mice with ascites cell tumors.

DISCUSSION

Four potential assays have been examined as possible alternatives or adjuncts to the standard incorporation of tritiated thymidine into DNA, as a measurement of lymphoblastic transformation in response to PHA stimulation. These assays included the measurement of cyclic GMP at 20 min, ^{33}P-phosphatidic acid at 30 min and cystathionine synthetase and uridine kinase at 24 hr after lymphocyte stimulation. These assays were compared to one another and to the standard tritiated thymidine incorporation assay which requires 3–7 days,

Table 1. In vitro *assay of PHA-stimulation of lymphocytes from tumor-bearing and immunosuppressed animals*

Source of lymphocytes	PHA (μg/ml)	Cyclic GMP (p-moles/10^6 cells)	^{33}P-phospholipid (counts/min per 10^6 cells)	Cystathionine synthetase	Uridine kinase (units/10^6 cells)	^{3}H thymidine incorporation (counts/min per 10^6 cells)
1. Normal rats	None	0·023±0·01	17± 3	143±15	320± 20	4180±200
	250	1·63 ±0·04	112±10	660±30	1330±100	68670±300
2. Immunosuppressed rats (40 mg/kg of Dexamethasone for 18 hr)	None	0·035±0·01		173±15	328± 20	4070±200
	250	0·059±0·01		184±15	406± 20	4980±250
3. Rats bearing Hepatoma 5123D	None	0·045±0·01	21± 3	117±15	301± 20	4280±200
	250	0·066±0·02	19± 3	232±20	442± 30	4230±200
4. Rats bearing Hepatoma 7800	None	0·031±0·01				
	250	0·040±0·01				
5. Adrenalextomized rats bearing Hepatoma 7800	None			151±15	344± 20	
	250			362±20	614± 40	

All experiments represent a minimum of three experiments ± standard deviation.

Table 2. *Effect of serum from normal and tumor-bearing animals on blastic transformation of lymphocytes from normal rats*

Source of serum	PHA (mg/ml)	Cyclic GMP (p-moles/10^6 cells)	Cystathionine synthetase (counts/min per 10^6 cells)	^{3}H Tdr incorporation into DNA (counts/min per 10^6 cells)
1. Normal rats	None	0·025±0·1	303±20	4177±200
	250	1·630±0·04	580±40	68672±300
2. S-37 bearing mice	None	0·04 ±0·01		
	250	0·62 ±0·02		
3. Hepatoma 7800 bearing rats	None		217±20	3300±200
	250		424±30	3440±200

All results represent a minimum of 3 experiments ± standard deviation.

depending upon the sensitivity desired and which shows considerable variation when lymphocytes from different, but apparently normal individuals are used. From the standpoint of practicality, sensitivity and possibly reliability, the cyclic GMP assay would appear to be the superior assay. However, measuring as it does one of the earliest biochemical events in lymphoblastic transformation requires that it be used in strictly defined conditions, as must also assays based on DNA synthesis, since neither represents the end product of the immune response. Such assays must be used in combination with assays for later events, preferably end-products, of the immune response particularly if defects may be present in the later phases of the afferent, or in the efferent arm of the immune response and particularly if the tests are being used for diagnostic purposes. However, a rapid sensitive assay might be useful in establishing immunological profiles, assessing the status of a patient during therapy, or testing the response of a patient's lymphocytes to specific tumor antigens.

The predictable suppression of the immune response by glucocorticoids [20] was employed here only as a means of comparing the *in vitro* blastic response by several assays under differ-

ent conditions of immune competence. However, the poor *in vitro* response of lymphocytes from animals bearing transplantable solid (i.e. Hepatomas 7800 and 5123 D) or ascites (S-37) tumors may be an important clue to the dependence of the progress of the disease or the host–tumor relationship. It follows that the tumor directly, or indirectly reduces the proportion of peripheral lymphocytes which are capable of responding to PHA. Although this was shown to be an inherent abnormality in the lymphocytes from tumor-bearing animals, the serum from the tumor-bearing animals also suppressed the transformation of lymphocytes from normal animals. The defective lymphocytes may be the result of immunological paralysis due to excessive tumor antigen in circulation [21] or to the related possibility that most of the cells are stimulated by the excess antigen to an advanced stage of differentiation where they are no longer sensitive to PHA [13]. The repressive effect of the tumor on the immune response is in accord with the results of clinical studies which show that some immunologically incompetent patients become competent after tumor regression following therapy [17]. Also in line with the present result is the observation [22] of the return of the immune response to rats bearing primary sarcomata following removal of the tumor.

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REFERENCES

1. H. L. Cooper, Studies on RNA metabolism during lymphocyte activation. *Transplant Rev.* **11,** 3 (1972).
2. B. H. Park and R. A. Good, A new micromethod for evaluating lymphocyte responses to phytohemagglutinin: quantitative analysis of the function of thymus-dependent cells. *Proc. nat. Acad. Sci. (U.S.A.)* **69,** 371 (1972).
3. D. B. Fisher and G. C. Mueller, Studies on the mechanism by which phytohemagglutinin rapidly stimulated phospholipid metabolism of human lymphocytes. *Biochim. biophys. Acta* **248,** 434 (1971).
4. S. A. Rosenberg and R. Levy, Synthesis of nuclear-associated proteins by lymphocytes within minutes after contact with phytohemagglutinin. *J. Immunol.* **108,** 1105 (1972).
5. J. W. Hadden, E. M. Hadden, M. K. Haddox and N. D. Goldberg, Guanosine 3′:5′-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. *Proc. nat. Acad. Sci. (USA)* **69,** 3024 (1972).
6. J. L. Goldstein, B. K. Campbell and S. M. Garterler, Cystathionine Synthetase activity in human lymphocytes: induction by phytohemagglutinin. *J. clin. Invest.* **51,** 1034 (1972).
7. M. G. Rosenfeld, I. B. Abass, J. M. Mendelsohn, B. A. Roos, R. F. Boone and L. D. Garren, Control of transcription of RNA rich in polyadenylic acid in human lymphocytes. *Proc. nat. Acad. Sci. (USA)* **69,** 2306 (1972).
8. J. E. Kay and S. D. Handmaker, Uridine incorporation and RNA synthesis during stimulation of lymphocytes by phytohemagglutinin. *Exp. Cell Res.* **63,** 411 (1970).
9. N. R. L. Bucher, M. N. Swaffield and J. F. Trioia, The influence of age upon the incorporation of thymidine-2-^{14}C into DNA of regenerating rat liver. *Cancer Res.* **24,** 509 (1964).
10. A. J. Rizzo and T. E. Webb, Regulation of Ribosome formation in regenerating rat liver. *Europ. J. Biochem.* **27,** 136 (1972).
11. G. Krystal and T. E. Webb, Multiple forms of uridine kinase in normal and neoplastic rat liver. *Biochem. J.* **124,** 943 (1971).
12. D. E. Schumm, H. P. Morris and T. E. Webb, Cytosol-modulated transport of messenger RNA from isolated nuclei. *Cancer Res.* **33,** 1821 (1973).
13. F. Daguillard, Immunologic significance of *in vitro* lymphocyte responses. *Med. Clin. North America* **56,** 293 (1972).
14. R. Christoforetti and P. Fireman, Studies of a non-cytotoxic migration inhibitory factor from human leucocytes. *Nature (Lond)* **241,** 219 (1973).

15. A. FEFER, Experimental approaches to immunotherapy of cancer. In *Current Concepts in the Management of Leukemia and Lymphoma* (Edited by J. E. Ultmann), Vol. 36 (Recent Results of Cancer Research, Heidelberg), Springer-Verlag, 1971, pp. 182-192.
16. A. R. CHEEMA and E. M. HERSH, Patient survival after chemotherapy and its relationship to *in vitro* lymphocyte blastogenesis. *Cancer* **28,** 851 (1971).
17. E. M. HERSH, J. P. WHITECAR, K. B. MCCREDIE, G. P. BODEY and E. J. FREIREICH, Chemotherapy, immunocompetence, immunosuppression and prognosis in acute leukemia. *New Engl. J. Med.* **285,** 1211 (1971).
18. R. H. MATTHEWS, C. A. LESLIE and P. G. SCHOLFEFIELD, Histidine uptake and exchange in S-37 ascites tumor cells. *Biochim. biophys. Acta* **203,** 457 (1970).
19. A. L. STEINER, C. W. PARKER and D. M. KIPNIS, The measurement of cyclic nucleotides by radioimmunoassay. *J. biol. Chem.* **247,** 1106 (1972).
20. S. SEGAL, I. R. COHEN and M. FELDMAN, Thymus-derived lymphocytes: humoral and cellular reactions distinguished by hydrocortisone. *Science* **175,** 1126 (1972).
21. M. C. RAFF, T and B lymphocytes and immune responses. *Nature (Lond.)* **242,** 19 (1973).
22. P. ALEXANDER, J. BENSTED and E. J. DELORME, The cellular immune response to primary sarcomata in rats. II. Abnormal responses of nodes draining the tumor. *Proc. royal Soc. Lond.* **174,** 237 (1969).

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The Effect of *Corynebacterium parvum* on the Structure and Function of the Lymphoid System in Mice

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Abstract—*The effects of* Corynebacterium parvum (C. parvum) *on the structure and function of the immune system of mice have been studied.*

C. parvum *pretreatment caused a fall of body weight and a significant decrease in thymic weight. It was associated with a slight increase of lymph node weight and considerable increase of liver and spleen weights.*

C. parvum *pretreatment was associated with prolongation of skin allograft survival and a slightly increased response to sheep erythrocytes; it did not affect the production of anti* H_2 *cytotoxic antibody.*

The percentage of θ-positive (T-cells) in the lymph nodes and spleens of mice given C. parvum *was less than untreated controls, and in the thymus the percentage of these cells was similar in the two groups of mice.*

The data presented suggests the C. parvum *treatment is associated with increased macrophage activity with concomitant depression of cell mediated immunity.*

INTRODUCTION

WITH THE demonstration that even autochthonous tumours excite an immune response in the subjects in which they arise, one approach to cancer therapy is non-specific potentiation of this response. During recent years many exogenous compounds have been suggested as agents capable of systemic stimulation of the immune system [1, 2, 3, 4]. *Corynebacterium parvum* (*C. parvum*) is known to have some effects on the immune system [5, 6], and it has powerful antitumour activity [7, 8, 9]. The effects of *C. parvum* on the structure and function of the immune system of mice has, therefore, been investigated.

METHODS

All mice were obtained from Jackson Laboratories Inc. and strains of CBA, A and C_3H were used in these experiments.

C. parvum was obtained from Wellcome Research Laboratories Batch EZ 174 and each dose constituted of 0·2 ml of a solution at 7 mg/ml wet weight given by intraperitoneal injection.

To investigate the effects of *C. parvum* on the size of lymphoid tissue two groups of male CBA mice aged six weeks were used. One group was given *C. parvum* on two consecutive days and the other was untreated. Eight mice were examined at the beginning of the experiment and subsequently eight mice from each group at weekly intervals for three weeks. At each examination mice were killed, the body weighed and the following lymphoid organs removed and weighed immediately; the thymus, pooled axillary and brachial lymph nodes, spleen and liver. The data were analysed by two way analysis of variance with interaction.

Skin graft survival was compared between male CBA mice aged three months given 0·2 ml *C. parvum* intraperitoneally either one day or one week before grafting and age matched, untreated control mice. Both groups were grafted using the method described by

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Billingham and Medawar [10]. Graft rejection was assessed by daily inspection of the grafts; when 75% of the graft was not viable, grafts were described as rejected. In one experiment mice were grafted with male A strain tail skin and in another male C_3H skin. At least 10 mice of each group were suitable for scoring of skin graft survival.

Response to 1×10^8 sheep erythrocytes (SRBC) in control of *C. parvum* pretreated mice was measured four days after antigen by observation of red cell lysis by spleen cells, using the methods of Cunningham [11]. To do this CBA mice aged eight weeks were given 0·2 ml *C. parvum* on two consecutive days and nine days later antigen was given.

Cytotoxic anti-H_2 antibody was measured in 10 male CBA mice aged three months after injection of 0·2 ml *C. parvum* intraperitoneally. Seven days later these mice and appropriate controls were skin grafted with male A strain tail skin and 14 days later all mice were tail bled and the serum from each group was pooled. Twenty days after first set grafting these same mice were regrafted with male A strain tail skin and they were tail bled 10 and 20 days later. Pooled sera from the three bleeds of both groups of mice were analysed for anti-H_2 cytotoxic activity using Ruszkiewicz' [12] modification of the Cr^{51} release method of Wigzell [13].

The percentage of θ-positive cells was determined by the tryan blue exclusion method [14] using anti-θ serum prepared in AKR mice by serial injection of thymocytes from CBA mice [14].

RESULTS

The average body weight of mice treated with *C. parvum* was always lower than controls and this difference was significant statistically (Table 1). However, the difference was only marked at two weeks after treatment. Mean thymus weight (Table 2) of mice given *C. parvum* reached a sustained minimum one week after treatment, and this value was considerably below the weight of control thymuses; when these thymus weights were corrected for body weight similar but greater differences between control mice and mice given *C. parvum* were seen.

The mean weight (Table 3) of pooled axillary and brachial lymph nodes was significantly heavier in mice which received *C. parvum* and when their weights were corrected for differences of body weight this difference was greater. The maximal difference of weight was seen one week after *C. parvum*, at two weeks it was less

Table 1. *Effects of two doses of* 0·2 *ml* C. parvum *on mean body weight of male CBA mice aged six weeks*

	Mean body weight (G) ± S.D.			
Time after injection	0	1 week	2 weeks	3 weeks
Control mice	19·02 ± 1·32	20·9 ± 0·68	20·7 ± 1·52	20·6 ± 1·47
Mice given *C. parvum*		19·7 ± 2·01	16·9 ± 2·12	19·6 ± 1·77

Table 2. *Effects of two doses of* 0·2 *ml.* C. parvum *on mean thymus weight of male CBA mice aged six weeks*

	Mean thymus weight (mg) ± S.D.			
Time after injection	0	1 week	2 weeks	3 weeks
Control mice	39·0 ± 9·77	41·4 ± 9·2	38·2 ± 10·6	45·0 ± 8·4
Mice given *C. parvum*		10·3 ± 5·4	10·9 ± 1·9	11·7 ± 2·8

Table 3. *Effects of two doses of* 0·2 *ml.* C. parvum *on mean lymph node weight of male CBA mice aged six weeks*

	Mean lymph node weight (mg) ± S.D.			
Time after injection	0	1 week	2 weeks	3 weeks
Control mice	8·7 ± 2·0	10·1 ± 3·2	10·3 ± 1·9	12·1 ± 0·9
Mice given *C. parvum*		15·1 ± 1·4	13·0 ± 3·3	12·3 ± 0·7

Table 4. *Effects of two doses of* 0·2 *ml* C. parvum *on mean spleen weight of male CBA mice aged six weeks*

	Mean spleen weight (mg) ± S.D.			
Time after injection	0	1 week	2 weeks	3 weeks
Control mice	79·5 ± 7·4	83 ± 7·7	79 ± 7·0	86 ± 8·4
Mice given *C. parvum*		358 ± 45·0	524 ± 84·0	614 ± 90·8

and then at three weeks lymph node weights of control and experimental mice were the same.

Mean spleen weight (Table 4) of mice given *C. parvum* increased progressively during the experiment so that three weeks after treatment the mean spleen weight of the experimental group was six times that of the controls. Similar but less change was seen in liver weight (Table 5). Three weeks after *C. parvum* mean liver weight of experimental mice was double that of controls. In the livers of some mice, pale areas of parenchyma were observed and histological examination of these showed zonal degeneration.

The effect of *C. parvum* pretreatment one week before grafting was to prolong graft survival (Fig. 1); the mean survival time of controls was 10·5 days and for experimental mice it was 19·5 days and in repeated experiments always showed increased graft survival in mice given *C. parvum* though prolongation was variable. Similar effects were noted when *C. parvum* was given on the day of grafting and when donor and recipient mice differed only at minor histocompatibility loci. For example, male CBA mice grafted with C_3H skin were given intraperitoneal *C. parvum* and the mean survival time of the allografts was 20 days compared with 15·5 days for untreated controls (Fig. 2).

C. parvum increased the direct response to SRBC when measured four days after antigen (Table 6).

The titre of cytotoxic antibodies to H_2 antigen was not significantly different between controls and *C. parvum* treated mice (Fig. 3). After first set grafting cytotoxic antibody was slightly higher in *C. parvum* treated mice; the serum dilution for killing 50% lymphocytes was between 1:32 and 1:64 and for controls it was between 1:16 and 1:32. Nine days after

Table 5. Effects of two doses of 0·2 *ml.* C. parvum *on mean liver weight of male CBA mice aged six weeks*

Time after injection	Mean liver weight (mg) ± S.D.			
	0	1 week	2 weeks	3 weeks
Control mice	115 ± 17·8	1100 ± 165	1278 ± 116	1408 ± 170
Mice given *C. parvum*		2146 ± 324	2037 ± 228	2503 ± 378

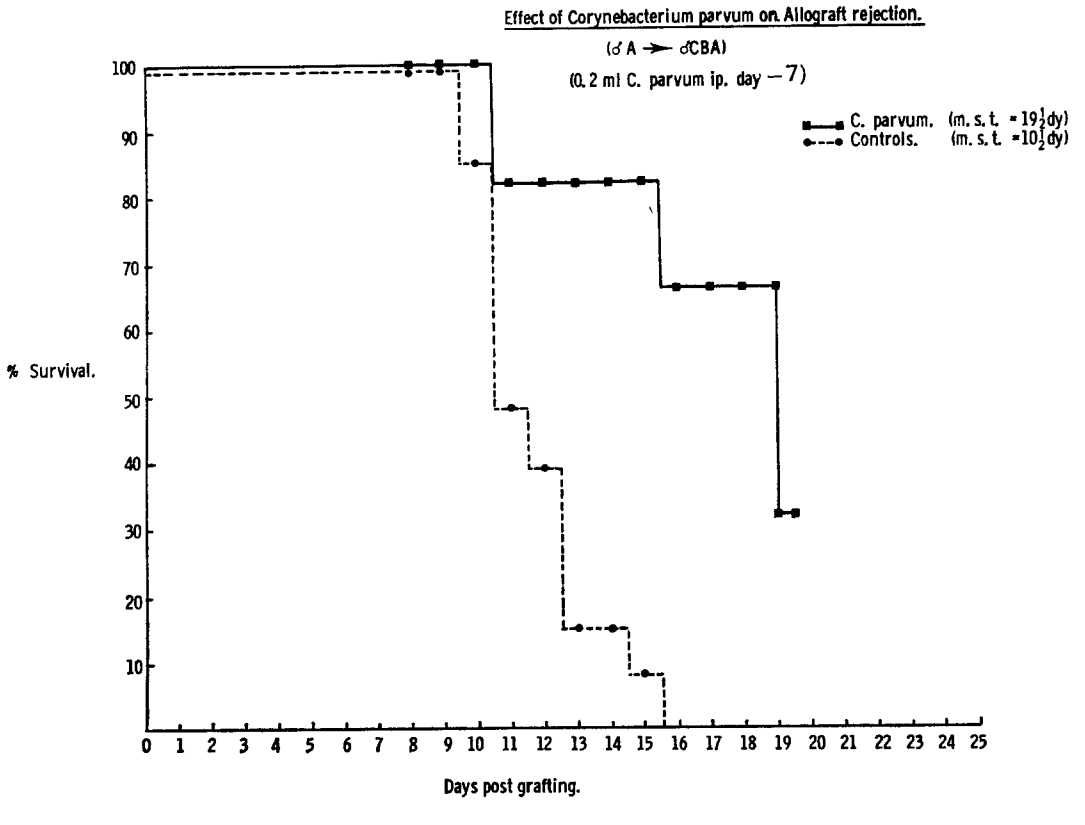


Fig. 1. Effect of C. parvum *pretreatment on skin allograft survival (A → CBA).*

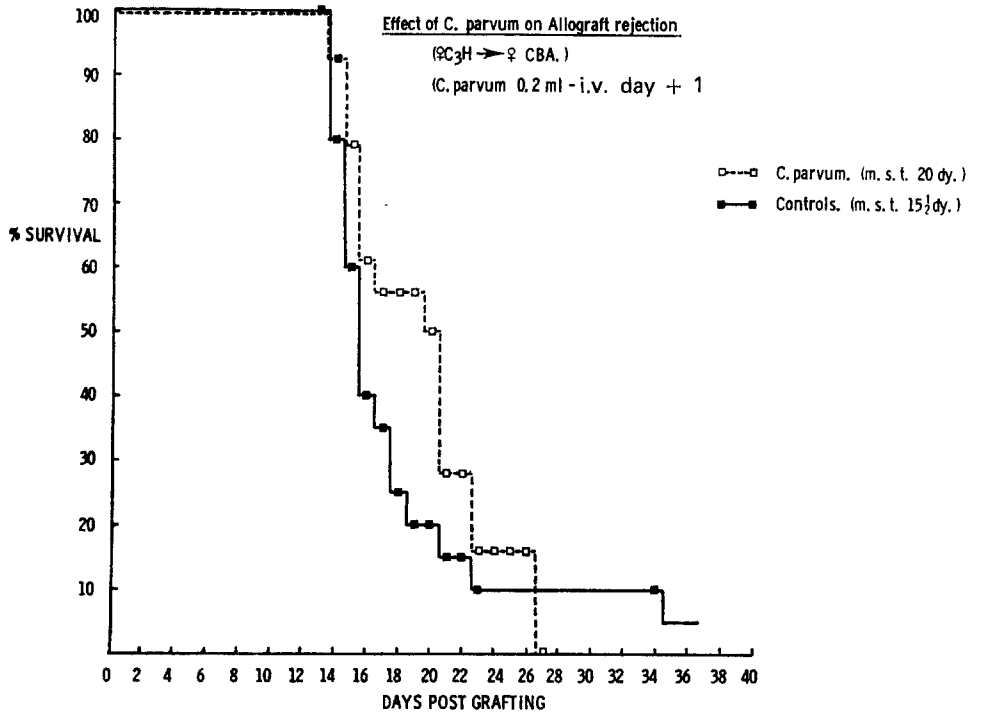


Fig. 2. Effect of C. parvum *on skin graft survival* ($C_3H \rightarrow CBA$).

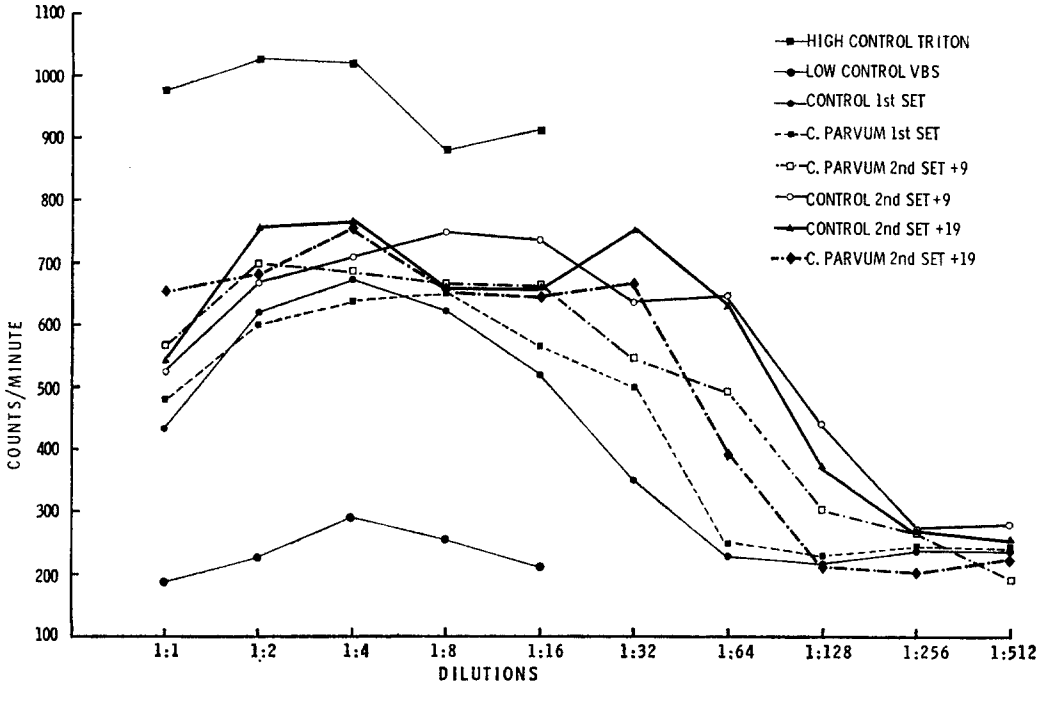


Fig. 3. Production of anti-H_2 cytotoxic antibody in C. parvum *treated and control mice measured after first and second set allografts. There was no significant difference between the groups (see text).*

Table 6. *Plaque forming cells/spleen in control and* C. parvum *treated mice*

	Control ($\times 10^3$)	*C. parvum* ($\times 10^3$)
	43·8	69·0
	18·5	34·4
	51·2	85·0
	48·9	81·0
	43·8	80·4
		70·1
Mean	41·2	69·9
S.D.	13·1	18·5
t-test	3·002	

second set grafting the antibody titre was higher in controls; 50% of lymphocytes were killed with control serum at a dilution of between 1:128 and 1:256 compared to between 1:64 and 1:128.

The percentage of cells killed by anti-θ serum in the lymph nodes and spleen of mice given *C. parvum* was less than untreated controls (Table 7) but in the thymus the percentage of these cells was similar in the two groups of mice.

mice given *C. parvum* and in untreated controls. However, after correction for differences of thymic size the absolute number of θ-positive cells in mice given *C. parvum* was significantly less.

These changes in the thymus were associated with depression of lymphocyte function. For example, there was prolongation of skin graft survival:— when *C. parvum* was given one week before grafting or on the day of grafting, and whether the donor and recipient were similar or different at the H_2 locus. In such experiments there was always prolongation of skin graft survival but the extent of this prolongation varied in different experiments. These results suggest depression of lymphocyte function after *C. parvum* and they are in agreement with the findings of Howard *et al.* [16].

C. parvum caused a slight increase of lymph node size but this was of short duration, and is unlikely to represent a change in the thymus dependent population of thymocytes for the percentage of θ-positive cells in the lymph nodes of mice given *C. parvum* was less than controls.

Table 7. *To show the % of cells lysed by anti-θ serum or normal mouse serum (NMS) in the lymphoid organs of control (Con) or* C. parvum *treated mice measured by the trypan blue exclusion test*

	Cytotoxicity					
	Lymph nodes		Thymus		Spleen	
	Con.	*C. parvum*	Con.	*C. parvum*	Con.	*C. parvum*
Untreated	0	1	2	2	2	1
N.M.S.	3	11	3	10	10	6
Anti θ	72	65	87	98	55	22

DISCUSSION

C. parvum pretreatment decreased thymic weight and this effect was magnified if allowance was made for lower body weights of mice given *C. parvum*. The mechanism of this change of thymus weight was not clear; it could reflect a specific effect on the thymus, or result from a non-specific effect of increased adrenal steroids due to stress. Attempts to elucidate this using adrenalectomised mice were unsuccessful, for adrenalectomised mice given *C. parvum* died. However, the findings of Scott [6, 15] suggest a specific effect for he found suppression of cell mediated immunity *in vitro*.

The content of θ-positive cells in the thymus was studied and it was found that the percentage of cells analysed by anti-θ was similarin

After *C. parvum* there was a significant increase of spleen and liver size. On histological examination this was due to increase of macrophages, and macrophage function, as assessed by increased clearance of carbon particles, was markedly increased (Castro; unpublished), which is in agreement with the findings of others [5]. In some mice given *C. parvum* there were areas of parenchymal liver damage and this was maximal in mice given *C. parvum* by the intravenous route. Such animals were abnormally sensitive to Nembutal anaesthesia which is detoxicated in the liver parenchyma. The percentage of cells lysed by anti-θ in the spleens of mice given *C. parvum* was less than appropriate controls. This could represent an actual decrease in this type of cell or a dilution of the particular cell population by other cells

(macrophages). When allowance was made for differences in the cell counts of lymphoid organs the absolute number of θ-positive cells from control mice and mice given *C. parvum* were similar.

The small increased response to sheep red blood cells in mice given *C. parvum* might reflect increased lymphocyte activity [17] or alteration of macrophage processing of this particular antigen. Certainly, there was no evidence of increased humoral antibody production when assessed by production of anti-H_2 cytotoxic antibody after skin allografting.

These data would therefore suggest that *C. parvum* is associated with increased macrophage activity with concomitant depression of lymphocyte function.

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REFERENCES

1. G. Biozzi, C. Stiffel, B. N. Halpern and D. Mouton, Effect de l'inoculation du bacille de Calmette Guerin sur le development de la tumour ascitique d'Ehrlich chez la souris. *C. R. Soc. Biol. (Paris)* **153,** 987 (1959).
2. W. Bollag, Prophylaxis of chemically induced benign and malignant epithelial tumours by vitamin A acid (retinoic acid). *Europ. J. Cancer* **8,** 689 (1972).
3. Y. Y. Maeda and G. Chihara, Lentinan, a new immune-accelerator of cell mediated responses. *Nature (Lond.)* **229,** 634 (1971).
4. J. C. Fisher, S. R. Cooperband and J. A .Mannick, The effects of Polyinosinic–Polycytidylic acid on the immune response of mice to antigenically distinct tumours. *Cancer Res.* **32,** 889 (1972).
5. B. N. Halpern, A. R. Prevot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard, Y. Bouthillier and C. Decreuseford, Stimulation de l'activité phagocytaire du système reticuloendothelial provaquee par *Corynebacterium parvum*. *J. reticuloendoth. Soc.* **1,** 77 (1964).
6. M. T. Scott, Biological effects of the adjuvant *Corynebacterium parvum*. I. Inhibition of PHA, mixed lymphocyte and GVH activity. *Cell. Immunol.* **5,** 459 (1972a).
7. 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).
8. S. E. Smith and M. J. Scott, Biological effects of *Corynebacterium parvum*: III. Amplification of resistance and impairment of active immunity to murine tumours. *Brit. J. Cancer* **26,** 361 (1972).
9. J. E. Castro, Antitumour effects of *Corynebacterium parvum* in mice. *Europ. J. Cancer* **10,** 121–127.
10. R. E. Billingham and P. B. Medawar, The technique of free skin grafting in mammals. *J. exp. Biol.* **28,** 385 (1951).
11. A. J. Cunningham, A method of increased sensitivity for detecting single antibody forming cells. *Nature (Lond.)* **207,** 1106 (1965).
12. S. V. Jooste, E. M. Lance, R. H. Levey, P. B. Medawar, M. Ruszkiewicz, R. Sharman and R. N. Taub, Notes on the preparation and assay of antilymphocytic serum for use in mice. *Immunology* **15,** 697 (1968).
13. H. Wigzell, Quantitative titrations of mouse H_2 iso-antibodies using Cr-51 target cells. *Transplantation* **3,** 423 (1965).
14. M. C. Raff, Theta isoantigen as a marker of thymus derived lymphocytes in mice. *Nature (Lond.)* **224,** 378 (1969).
15. M. T. Scott, Biological effects of the adjuvant *Corynebacterium parvum*: II. Evidence for macrophage-T cell interaction. *Cell Immunol.* **5,** 465 (1972b).
16. J. G. Howard, M. T. Scott and G. H. Christie, Cellular mechanisms underlying the adjuvant activity of *Corynebacterium parvum*: interaction of activated macrophages with T and B lymphocytes. In *Immunopotentiation.* (Edited by Julie Knight). Associated Scientific Publishers, Amsterdam (1973).
17. J. H. L. Playfair, Cell cooperation in the immune response. *Clin. exp. Immunol.* **8,** 839 (1971).

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Antitumour effects of *Corynebacterium parvum* in Mice

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Abstract—Corynebacterium parvum (C. parvum) *pretreatment decreased death rate of B alb/c mice inoculated with Meth A ascitic tumour, CBA mice given S37 ascitic tumour and C_3H/He mice given Ehrlich's ascitic tumour.*

Intravenous C. parvum *did not affect the death rate from ascitic Meth A tumour and intraperitoneal* C. parvum *did not affect the growth of solid Meth A tumour.*

The protective effects of intraperitoneal C. parvum *on ascitic Meth A tumour was maximal with low tumour dose and the duration of effect was between* 25 *and* 50 *days. Splenectomy did not affect the antitumour activity.*

The findings that previous thymectomy or antilymphocyte serum did not affect the antitumour activity of C. parvum *suggest that its action is not mediated through a change of cell mediated immunity.*

INTRODUCTION

RESISTANCE to tumours in laboratory animals parallels reticuloendothelial phagocytic activity [1] and during growth of transplanted, syngeneic tumours there is increased production [2] and function of macrophages [3–5] which reflects changes in cellular factors rather than opsonins [5]. Administration of *Corynebacterium parvum* (*C. parvum*) increases the number of macrophages and their function, and it protects against some tumours [6–8]. The experiments to be described investigate further the antitumour activity of *C. parvum* and the mechanism by which antitumour activity is effected.

METHODS AND MATERIALS

All mice were males from Jackson Laboratories Inc. and Balb/c, CBA and C_3H/He strains were used in these experiments.

C. parvum was obtained from Wellcome Research Laboratories, batch EZ 174 and two consecutive doses of 0·2 ml of a solution at 7 mg/ml were given immediately before tumour inoculation.

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In most tumour transplantation experiments a methylcholanthrane induced sarcoma (Meth A) obtained from, and described by Old *et al.* [9] was used. It has been maintained in our laboratory in ascitic form by weekly passage of 0·1 ml of malignant ascites into recipient Balb/c mice. We have confirmed that the tumour is syngeneic for Balb/c mice, it is antigenic, exhibits a good dose/response curve and electron microscopy of the tumour did not show viral particles. Intraperitoneal tumour challenge was by $3{\cdot}5 \times 10^3$ viable tumour cells in 0·1 ml Eagles medium and subcutaneous challenge (so that the tumour grew in solid form) was by $3{\cdot}5 \times 10^3$ cells in 0·05 ml Eagles medium. Death with tumour was recorded daily.

In a single experiment 1×10^5 S37 sarcoma cells were used and were maintained by weekly intraperitoneal passage into CBA mice, and in another 1×10^5 Ehrlich's ascitic tumour cells maintained in C_3H/He mice were used.

Thymectomy was by modifications of the methods described by Miller in 1967 [10]. Splenectomy was through an anterolateral, subcostal incision; splenic vessels were ligated and transected. Skin and body all were closed by a single suture [10].

Mouse antithymocyte serum was prepared

in sheep [11] and 0·25 ml was given subcutaneously for three days before tumour inoculation and thereafter a single subcutaneous injection of 0·25 ml at weekly intervals.

To study the effect of *C. parvum* pretreatment on Meth A tumour, Ehrlich's tumour and S37 sarcoma groups of 20 mice were used. In each experiment one group of mice was given *C. parvum* intraperitoneally and 24 hr later these mice and a group of untreated control mice were inoculated with tumour intraperitoneally.

To compare the antitumour activity of *C. parvum* given intravenously and intraperitoneally, three groups of 12 mice were used. One group received *C. parvum* by intravenous injection and another received *C. parvum* by intraperitoneal injection; a third group was untreated. Twenty-four hours after the last dose of *C. parvum* all three groups were given tumour cells intraperitoneally.

To test the effect of *C. parvum* on solid Meth A tumour 10 mice received intraperitoneal *C. parvum* and 10 untreated litter mates were controls. Twenty-four hours later all mice were injected subcutaneously in the interscapular region with tumour cells so that the tumour grew in solid form. All mice were examined twice weekly and palpable tumours were measured; when tumours were 3·0 cm in maximal diameter such mice were killed and recorded as tumour death.

The antitumour effect of *C. parvum* on different tumour doses was studied. Six groups of at least eight mice were given intraperitoneal *C. parvum* and two weeks later these and appropriate controls were given graded doses of Meth A tumour cells ($3{\cdot}5 \times 10^2$, 10^4, 10^6, 10^7, 10^8) intraperitoneally and death with tumour recorded.

The duration of antitumour activity was established using five groups of 12 mice. Four groups were given *C. parvum* intraperitoneally and one group was untreated. Each of the four treated groups was given the *C. parvum* at a different interval (2, 25, 50 and 100 days) before intraperitoneal inoculation with tumour cells.

Because *C. parvum* administration is associated with splenomegally [12, 13] the effect of splenectomy on the antitumour activity of *C. parvum* was studied. Four groups of 20 mice were used and two of these were splenectomised. One splenectomised group and one untreated group received *C. parvum* intraperitoneally and the remaining two groups were untreated. Two weeks later all four groups were injected with tumour cells intraperitoneally.

The possibility of *C. parvum* being effective through a cell mediated immune pathway was investigated by combining *C. parvum* treatment with thymectomy or antilymphocyte serum. The effect of thymectomy on the antitumour activity of *C. parvum* was studied in four groups of 20 mice. Two groups were thymectomised and two were not. Two weeks later both the thymectomised groups and one of the controls were given *C. parvum*; one of the thymectomised groups given *C. parvum* received no further treatment in order to see if *C. parvum* is toxic in such mice. The remaining two control groups (one of which received *C. parvum*) and the remaining thymectomised group given *C. parvum* were all injected intraperitoneally with tumour cells.

In the study with A.L.S. five groups of mice were used, as follows:

(i) Normal mice given tumour cells
(ii) Normal mice given A.L.S. and tumour cells
(iii) Mice given *C. parvum* and tumour cells
(iv) Mice given *C. parvum*, A.L.S. and tumour cells
(v) Mice were given *C. parvum* and A.L.S.

C. parvum was given intraperitoneally for two days immediately after the initial course of A.L.S. and it was followed 24 hr later by tumour cells.

RESULTS

The effect of *C. parvum* treatment on three ascitic tumours, Meth A, S37 and Ehrlich's sarcoma was studied.

The effect of *C. parvum* on the growth of Meth A cells in Balb/c mice was to decrease the rate of death with tumour so that no experimental mice died during the duration of the experiment (Table 1). *C. parvum* also delayed death with tumour in CBA mice given S37 sarcoma (Table 2) and similar results were observed in C_3H/He mice given Ehrlich's ascitic tumour (Table 3).

When intravenous *C. parvum* was given, death rate from intraperitoneal Meth A was similar to untreated controls (Table 4) and similarly when intraperitoneal *C. parvum* was given it had no protective effect on a solid tumour (Table 5).

The protective effects of intraperitoneal inoculation of *C. parvum* on ascitic Meth A tumour depended upon the dose of tumour cells. At high tumour dose (10^8) the protective effect was small but with lower tumour dose the protective effects increased so that a good protection was observed with an inoculation of 10^2 cells (Fig. 1).

Table 1. *Percentage survival of control and* C. parvum *treated mice after* $3{\cdot}5 \times 10^3$ *Meth A tumour cells intraperitoneally.* C. parvum 0·2 *ml was given for two days* 24 *hr before intraperitoneal tumour inoculum and controls were untreated*

Treatment	Percentage survival after tumour at		
	25 days	30 days	35 days
Controls	90	50	15
C. parvum	100	100	100

Table 2. *Percentage survival of control and* C. parvum *treated mice after* 1×10^5 *S37 tumour cells given intraperitoneally.* 0·2 *ml* C. parvum *was given on two consecutive days before intraperitoneal tumour inoculation*

Treatment	Percentage survival after tumour at		
	20 days	30 days	40 days
Controls	30	5	5
C. parvum	85	15	10

Table 3. *Percentage survival of control and* C. parvum *treated mice after* 1×10^5 *Ehrlich's sarcoma cells given intraperitoneally.* 0·2 *ml* C. parvum *was given on two consecutive days before intraperitoneal tumour inoculation*

Treatment	Percentage survival after tumour at		
	15 days	20 days	25 days
Controls	100	50	0
C. parvum	100	100	100

Table 4. *Percentage survival of controls or mice given* C. parvum *by intraperitoneal or intravenous injection on death from* $3{\cdot}5 \times 10^3$ *Meth A tumour in cells oculated intraperitoneally*

Treatment	Percentage survival after tumour at		
	25 days	30 days	35 days
Controls	100	48	32
I.V. *C. parvum*	80	35	28
I.P. *C. parvum*	100	100	90

Table 5. *Percentage survival of* C. parvum *and control mice inoculated with* $3{\cdot}5 \times 10^3$ *Meth A tumour cells subcutaneously so that the tumour grew in solid form*

Treatment	Percentage survival after tumour at		
	30 days	50 days	70 days
Controls	100	53	48
C. parvum	68	46	24

Table 6. *Percentage survival of controls and mice given* C. parvum *to show the effects of increasing interval between treatment with* C. parvum *and challenge with* $3{\cdot}5 \times 10^3$ *ascitic Meth A tumour cells. When tumour was given two and* 25 *days after* C. parvum *there was a protective effect of* C. parvum *but at* 50 *days this was lost for the death rate in these groups was similar to that of control mice not given* C. parvum

Treatment	Percentage survival after tumour at		
	30 days	45 days	60 days
Control	80	5	0
C. parvum 50 days after tumour	65	15	15
C. parvum 25 days after tumour	100	25	15
C. parvum 2 days after tumour	92	60	33

Table 7. *Percentage survival of control and* C. parvum *treated mice to show the effects of splenectomy on the antitumour effect of* C. parvum

Treatment	Percentage survival after tumour at		
	30 days	45 days	60 days
Control	90	73	59
Control + splenectomy	65	45	38
C. parvum + splenectomy	95	95	68
C. parvum	100	95	81

Table 8. *Percentage survival of control and* C. parvum *treated mice to show the effect of thymectomy on the antitumour activity of* C. parvum. *Control mice given tumour died rapidly and* C. parvum *given to the control mice had a protective effect. Thymectomy only slightly inhibited this protective effect*

Treatment	Percentage survival after treatment at		
	20 days	30 days	40 days
Control + tumour	100	60	14
C. parvum + tumour	100	100	100
Thymectomy + *C. parvum* + tumour	100	100	84
Thymectomy + *C. parvum*	100	100	100

Table 9. *Percentage of controls and* C. parvum *treated mice to show the effect of A.L.S. on the antitumour activity of* C. parvum. C. parvum *was given on days* 1 *and* 2, *and A.L.S. on days* 3, 4, 5 *and then weekly. Tumour cells were given intraperitoneally on day* 0

Treatment	Percentage survival after treatment at		
	30 days	40 days	50 days
Control + tumour	92	55	45
Control, A.L.S. + tumour	66	8	0
C. parvum, A.L.S. + tumour	83	83	75
C. parvum + tumour	100	100	100
C. parvum + A.L.S.	92	75	50

Duration of the antitumour effect of *C. parvum* was established by giving intraperitoneal *C. parvum* at intervals before intraperitoneal tumour inoculation. When the interval was two days *C. parvum* afforded good protection. When tumour was given 25 days after *C. parvum* there was still good protection but it was less than that observed at two days. When the drug–tumour interval was increased to 50 or 100 days a protective effect was not seen (Table 6).

Splenectomy had only slight effect on the antitumour acitivity of *C. parvum* (Table 7) and splenectomised mice given *C. parvum* survived considerably longer than untreated controls. Splenectomised control mice died faster than untreated control mice and, therefore, considerably faster than splenectomised mice given *C. parvum.*

When thymectomy was combined with *C. parvum* treatment, this combination of treatment was not lethally toxic for all thymectomised mice given *C. parvum* survived for the duration of the experiment (40 days) (Table 8). Control mice given tumour died rapidly so that only 10% of these were alive at 40 days but *C. parvum* had a protective effective for every control mouse given *C. parvum* survived after tumour inoculation. Thymectomy had only a very slight inhibitory effect on protection by *C. parvum.*

When A.L.S. was given to control mice they died rapidly with tumour and all of them were dead by 42 days (Table 9). Control, untreated mice survived for longer than the A.L.S. treated group for at 50 days after tumour inoculations five of 11 were alive. Mice given *C. parvum* alone were protected from tumour, all of them surviving for the duration of the experiment. When A.L.S. was given to mice treated with *C. parvum* the antitumour effect of *C. parvum* was slightly inhibited but this could have been the result of non-specific toxicity of A.L.S. in *C. parvum* treated mice which was shown to occur.

DISCUSSION

In well defined circumstances *C. parvum* has powerful antitumour activity and the antitumour effects of intraperitoneal *C. parvum* against the ascitic Meth A tumour in Balb-C mice, Ehrlich's tumour in C_3H/He mice and S37 sarcoma in CBA mice have been shown. When *C. parvum* was given intraperitoneally and tumour inoculum injected subcutaneously protection was not afforded and neither was it afforded when intravenous *C. parvum* was given before intraperitoneal tumour inoculation. Certainly with other solid tumours protection has been afforded but in the experiment with solid tumour described, mice pretreated with *C. parvum* in fact fared slightly worse than the untreated controls and the possibility of tumour enhancement requires further investigation.

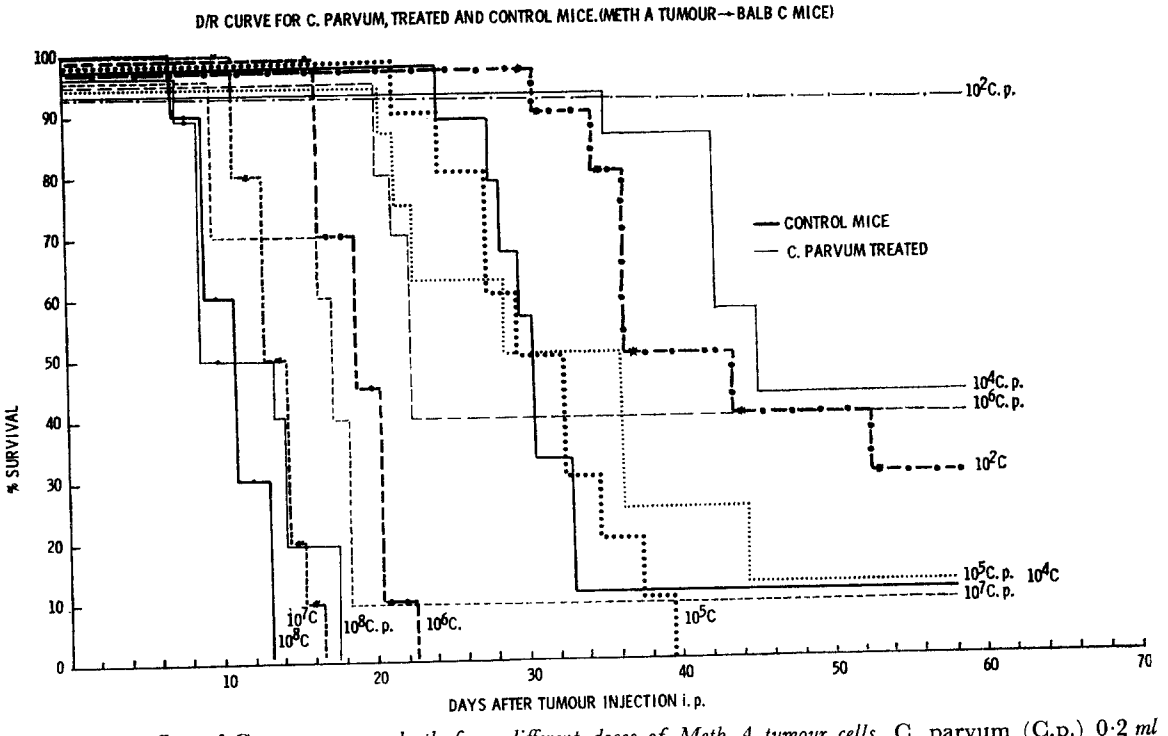


Fig. 1. *The effect of* C. parvum *on death from different doses of Meth A tumour cells.* C. parvum (C.p.) 0·2 *ml was injected intraperitoneally on two consecutive days and tumour given intraperitoneally two weeks later to these and appropriate controls* (*C*).

At high tumour dosage (10^8) the protective effects of *C. parvum* were minimal, but with lower tumour dosage there was progressive protection and this was maximal at the lowest tumour dose (10^2 cells) and some mice given *C. parvum* survived indefinitely. For example, three of 10 untreated mice (controls) had survived 70 days (when the experiment was terminated) after inoculation with $3{\cdot}5 \times 10^2$ cells, whereas all 10 of the mice given *C. parvum* survived and were tumour free. Furthermore, these 10 mice have now survived 150 days without developing tumour and may be regarded as permanently cured of tumour.

The duration of antitumour activity against Meth A ascitic tumour was between 25 and 50 days. When the interval between *C. parvum* treatment and inoculation with tumour was two days good protection was afforded; when this interval was 25 days there was still protection but it was less, and when the interval was 50 or 100 days protection was not afforded.

Administration of *C. parvum* is associated with increased phagocytic activity. Halpern *et al.* [13] showed increased clearance of carbon particles and there is enlargement of the liver and spleen which on histological examination was due to increased macrophages. However, splenectomy only slightly inhibited the antitumour activity of *C. parvum* but increased macrophages at other remaining sites (liver, lymph nodes, peritoneum) could explain this. It is interesting to note that splenectomy of normal Balb/c mice increased death rate, possibly by removal of fixed macrophages or by reducing cytotoxic antibody. This finding contrasts with the observations of Hellström [14] who reported protection against tumour death after splenectomy possibly because of less enhancing antibody. In such experiments, details particularly with regard to timing and tumour dose may be important in the balance between cytotoxicity and enhancement.

It is well recognized that cell mediated immunity is important in the destruction of some tumours. The relationship between the antitumour activity of *C. parvum* was investigated in thymectomised mice and mice given A.L.S. When thymectomy was combined with *C. parvum* pretreatment there was only slight, late inhibition of antitumour activity; control mice given *C. parvum* survived throughout the experiment whereas a small percentage of thymectomised mice given the drug died from tumour at 30 days. It could be that a subpopulation of thymus deprived cells, which are long-lived cells (> three weeks) [15] were required for the protective effects of *C. parvum;* failure to replace these after thymectomy resulting in loss of the antitumour effect of *C. parvum.* Alternatively, as the antitumour activity of *C. parvum* declined (between 25 and 50 days) thymectomised mice were at a disadvantage compared to controls.

The relationship between antitumour activity of *C. parvum* and cell mediated immunity was further investigated by combining *C. parvum* with A.L.S. A.L.S. in the dosage used in these experiments has a selective effect upon cell mediated immunity [16]. In such experiment A.L.S. caused slight reduction of antitumour activity of *C. parvum*, but this could have been the result of non-specific toxicity of A.L.S. in *C. parvum* treated mice which was seen to occur. Furthermore, despite A.L.S., *C. parvum* was still a very active antitumour agent for mice given these two agents survived considerably longer than normal mice given A.L.S. and also better than normal mice after tumour inoculation.

It would seem unlikely that changes in lymphocyte activity were implicated in the antitumour activity of *C. parvum* for it was retained in thymectomised mice or A.L.S. treated mice which were deficient in cell mediated immunity. These observations agree with those of Woodruff *et al.* [17] using a different experimental model. Whilst not excluding the necessity of a small, limiting number of cells responsible for cell mediated immunity these data suggest that the increased antitumour activity of *C. parvum* treated mice is not related to increased thymus processed cells. It is most likely that the antitumour activity of *C. parvum* is related to increased macrophage function but the mechanism underlying these observations is not clear. There is morphological evidence from electron microscopic studies of macrophage: lymphocyte interaction at the onset of immune responses [18] and it is accepted that macrophages process antigens for lymphocytes [19] but they have other functions which may be important. Macrophage mediated cytotoxicity to tumour cells has been described [20]; macrophages are essential in the responses to phytohaemagglutinin [21] and they may play an important part in maintaining a balance between cellular immunity and enhancement by removing excess antigen [22, 23] or complexes [24] from the circulation and such possibilities require further investigation.

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REFERENCES

1. L. J. Old, B. Benacerraf, D. A. Clarke, E. A. Carswell and E. Stockert. The role of the reticuloendothelial system in host reaction to neoplasia. *Cancer Res.* **21,** 1281 (1961).
2. M. Baum and B. Fisher. Macrophage production by the bone marrow of tumour bearing mice. *Cancer Res.* **32,** 2813 (1972).
3. L. J. Old, D. A. Clarke, B. Benacerraf and M. Goldsmith. The reticuloendothelial system and the neoplastic process. *Ann. N. Y. Acad. Sci.* **88,** 264 (1960).
4. R. W. Blamey. Experiments in tumour immunology. *Brit. J. Surg.* **55,** 769 (1968).
5. R. F. Klampschmidt and L. A. Pulliam. Changes in the opsonin and cellular influences on phagocytosis during the growth of transplantable tumours. *J. reticuloendoth. Soc.* **11,** 1 (1972).
6. 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).
7. L. H. Smith and M. F. A. Woodruff. Comparative effects of two strains of *Corynebacterium parvum* on phagocytic activity and tumour growth. *Nature (Lond.)* **219,** 197 (1968).
8. S. E. Smith and M. J. Scott. Biological effects of *Corynebacterium parvum: III.* Amplification of resistance and impairment of active immunity to murine tumours. *Brit. J. Cancer* **26,** 361 (1972).
9. L. J. Old, E. A. Boyse, D. A. Clarke and E. A. Carswell. Antigenic properties of chemically induced tumours. *Ann. N. Y. Acad. Sci.* **101,** 80 (1962).
10. J. E. Castro. Surgical procedures in small laboratory animals. (In press) (1974a).
11. R. M. Binns, E. Simpson, S. Nehlsen and M. Ruszkiewicz. A new species for the production of strongly immunosuppressive antilymphocyte sera. In *International Symposium on Antilymphocyte Serum, Versailles,* 1970. *Symposia Series Immunobiological Standardization,* **16,** 97. Karger (1970).
12. J. E. Castro. The effect of *Corynebacterium parvum* on the structure and function of the lymphoid system in mice. *Europ. J. Cancer* **10,** 115–120. 1974b.
13. B. N. Halpern, A. R. Prevot, G. Biozzi, C. Stiffel, D. Mouton, J. C. Morard, Y. Bouthillier and C. Decreuseford. Stimulation de l'activite phagocytaire du systeme reticuloendothelial provaquee par *Corynebacterium parvum. J. reticuloendoth. Soc.* **1,** 77 (1964).
14. K. E. Hellström, and I. Hellström. Cellular immunity against tumour antigens. *Adv. Cancer Res.* **12,** 167 (1969).
15. M. C. Raff and H. Cantor. Subpopulations of thymus cells and thymus-derived lymphocytes. *Progress in Immunology.* (Edited by Bernard Amos) Academic Press (1971).
16. J. G. Gray, A. P. Monaco, M. L. Wood and P. S. Russell. Studies on heterologous anti lymphocyte serum in mice I. *In vitro* and *in vivo* properties. *J. Immunol.* **96,** 217 (1966).
17. M. F. A. Woodruff, N. Dunbar and A. Ghaffar. The growth of tumours in T cell deprived mice and their response to treatment with *Corynebacterium parvum. Proc. roy. Soc. B.* **184,** 97 (1973).
18. S. B. Salvin, S. Sell and J. Nishio. Activity *in vitro* of lymphocytes and macrophages in delayed hypersensitivity. *J. Immunol.* **107,** 655 (1971).
19. B. A. Askonas and J. M. Rhodes. Immunogenicity of antigen containing ribonucleic acid preparations from macrophages. *Nature (Lond.)* **205,** 470 (1965).
20. R. Evans and P. Alexander. Mechanisms of immunologically specific killing of tumour cells by macrophages. *Nature (Lond.)* **236,** 168 (1972).
21. W. R. Levis and J. H. Robbins. Effect of glass adherent cells on the blastogenic response of 'purified' lymphocytes to phytohaemagglutinin. *Exp. Cell Res.* **61,** 153 (1970).

22. E. R. Unanue and J. C. Cerottini. The immunogenicity of antigen bound to the plasma membrane of macrophages. *J. exp. Med.* **131,** 711 (1970).
23. J. Proctor, C. M. Rudentam and P. Alexander. Increased incidence of lung metastases following treatment of rats bearing hepatomas with irradiated tumour cells and the beneficial effect of *Corynebacterium parvum*. *Biomedicine* **19,** 248 (1973).
24. G. Haughton. Specific immunosuppression by minute doses of passive antibody. *Cell Immunol.* **2,** 567 (1971).

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A Sex-Dependent Difference in the Type of Tumours Induced by Dimethylnitrosamine in the Livers of C3Hf Mice

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Abstract—*Tumour formation in adult C3Hf mice by dimethylnitrosamine was studied. Administration of DMNA for* 13 *weeks (total doses* 112.4–122.5 *mg/kg, in drinking water) to males and females resulted in a sex-dependent response, males obtaining mainly hepatomas and females predominantly vascular tumours in both the liver and at other sites. Vascular lesions in the livers of female mice were classified as peliosis, capillary hemangioma, cavernous hemangioma as well as hemangioendothelioma. Hepatoma incidence in males was significantly increased after a single injection of DMNA (7 mg/kg, intraperitoneally) into the adult animal; this treatment had no effect on tumor incidence in female mice. Non-neoplastic changes in a variety of lymph nodes were found in* 99-*weeks old control females and resembled so-called mesenteric disease in which the mesenterial lymph node is involved mainly. This non-neoplastic disease was neither observed in untreated* 58-*weeks old females nor in DMNA-treated females of the same age.*

INTRODUCTION

STRIKING sex-dependent differences in liver-tumour incidences in mice or rats after giving carcinogens like dimethylnitrosamine [1], N-hydroxy-acetylaminofluorene [2], *ortho*-aminoazotoluene [3], aflatoxin B_1 [4], and N–4–(4′–fluorobiphenyl) acetamide [5] have been reported.

In order to extend our earlier investigations [6] on the role of DNA methylation in the process of tumourigenesis by DMNA, C3Hf-male and -female mice were tested for their susceptibility to tumour formation (especially in the liver) by this nitrosamine.

MATERIAL AND METHODS

Carcinogen

DMNA was supplied by Aldrich Chemical Co. (Milwaukee, Wis.), distilled before use and kept in the dark at −20°C.

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Abbreviation: DMNA, dimethylnitrosamine

Animals

Housing conditions and diet of C3Hf (C3Hf/HeAf) mice were the same as described previously [6]. During the experiment the animals were subjected to a strict light–dark regimen (start of the 11-hr light period at 8.00 a.m.).

Tumour experiments

C3Hf-male and -female mice were divided into nine groups (4 males, 5 females, see Table 1), each group containing at least 18 animals. At the age of 11–13 weeks one group of each sex was injected with DMNA (7 mg/kg intraperitoneally). Starting at the same age, one male and two female groups received DMNA in their drinking water (10 ppm, renewed thrice weekly) during the succeeding 13 weeks followed by normal drinking water until death. Two male and two female groups were left untreated to serve as control groups. With the exception of two control groups (one male, one female) which were sacrificed at the age of 99 weeks, all remaining animals in the other

Table 1. Tumours in the liver of C3Hf-male and -female mice after DMNA administration

Group	1	2	3	4	5	6	7	8	9
Sex	♂	♂	♂	♂	♀	♀	♀	♀	♀
Treatment	Control	Control	DMNA*	DMNA†	Control	Control	DMNA*	DMNA†	DMNA†
Total dose of DMNA (mg/kg)	0	0	7·0	114·1	0	0	7·0	112·4	122·5
Number of animals at the start of the experiment	50	19	19	18	20	20	20	20	20
End of the experiment (age in weeks)	58	99	58	58	58	99	58	58	58
Number of animals examined	50	19	18	17	20	20	20	20	20
Number of animals dead within 13 weeks after start	0	0	1(5%)	0	0	0	0	1(5%)	1(5%)
Number of animals dead before the end of the experiment	1(2%)	1(5%)	1(5%)	3(17%)	1(5%)	9(45%)‡	0	20(100%)‡	20(100%)‡
Mean survival time (age) in weeks)	58	99	56	58	57	94	58	51	44
Number of liver tumour bearing animals	6(12%)	6(31%)	7(38%)	10(59%)	1(5%)	0(0%)	0(0%)	9(45%)	12(60%)
Hepatoma§	6(12%)	6(31%)	7(38%)	7(41%)	1(5%)	0	0	1(5%)	0
Capillary hemangioma§	0	0	0	3(18%)	0	0	0	7(35%)	7(35%)
Cavernous hemangioma§	0	0	0	0	0	0	0	3(15%)	6(30%)
Hemangioendothelioma§	0	0	0	0	0	0	0	1(5%)	5(25%)

* One intraperitoneal injection of 7 mg DMNA/kg.
† 13 weeks DMNA in drinking water (10 ppm).
‡ See Table 2 for further survival data.
§ Number (percentage) of animals bearing this particular kind of tumour in the liver.

groups were killed at the age of 58 weeks. In some groups a number of animals were found dead or had to be killed when moribund before the end of the experimental period. Group 1 consisted of two subgroups of 20 and 30 animals respectively, the second subgroup being started about 4 months after termination of the first one.

Morphological examination

At autopsy all animals were inspected for tumours and other lesions. All lesions (except most lung tumours and some renal lesions) were routinely stained with hematoxylin-azophloxin and examined microscopically. Tumours of the lung were counted with the aid of a dissecting microscope (magnification × 10); only nodules with a diameter of 0·1 mm or larger were counted.

RESULTS

Tumour formation in male and female C3Hf mice

Survival. Mean survival times, listed in Table 2, showed that in most groups the survival was high. Only groups 6, 8 and 9 had appreciable

Table 2. Survival in DMNA-treated and control C3Hf-female mice

Group	Treatment	Number of survivors (age in weeks)*								
		20	30	40	50	60	70	80	90	100
5†	untreated control	20	20	19	19	0‡				
6	untreated control	20	20	20	20	20	20	18	15	0§
7	1 × DMNA	20	20	20	20	0‡				
8	13 weeks DMNA	19	19	19	8	0‡				
9	13 weeks DMNA	20	19	12	5	0‡				

* Each group started with 20 animals.
† For further details, see Table 1.
‡ End of the experiment at 58 weeks.
§ End of the experiment at 99 weeks.

numbers of animals which died or had to be killed before the end of the experiment. Further survival data for these groups are given in Table 2. In C3Hf females treated for 13 weeks with DMNA in drinking water many animals (group 8: 10/20; group 9: 12/20) died or had to be killed because of haemorrhagic ascites (up to a total volume of about 10 ml in some animals), only in two animals clear, non-haemorrhagic fluid was found in the abdominal cavity. Groups 8 and 9 differed in the onset of the rapid decline in survival (in group 8 starting at 40 weeks, in group 9 at 30 weeks), this might be the result of the small difference in daily dose of DMNA.

Tumours and other lesions in the liver

Tumours in the liver are recorded in Table 1. With the exception of three animals in group 4 (13 weeks DMNA in drinking water), all tumours in the liver of C3Hf males proved to be hepatomas histologically. These hepatomas did not structurally differ from those described in the literature [7] and should be regarded as low grade malignant tumours [8, 9]. In comparison with untreated controls (group 1) hepatoma incidences were significantly increased in group 3 after one DMNA injection of 7 mg/kg ($0{\cdot}010<P<0{\cdot}025$, χ^2-test) and in group 4 after 13 weeks DMNA in drinking water ($0{\cdot}010<P<0{\cdot}025$, χ^2-test). Hepatoma incidence in C3Hf females was found to be very low; in group 5 (control, 58 weeks) and group 8 (13 weeks DMNA, 58 weeks) one hepatoma was found in each group. Lesions in the liver of C3Hf females mainly consisted of vascular lesions, including peliosis, capillary and cavernous hemangiomas as well as malignant hemangioendotheliomas [cf. 10]. A single injection of DMNA had no effect on the tumour incidence in female mice, in contrast to the situation in the C3Hf male. Peliosis was found in female mice treated for 13 weeks with DMNA, one animal in group 8 and three animals in group 9 showed this lesion which consists of multiple cystic spaces of various sizes filled with blood (Fig. 1). The histological picture is similar to human and murine [11] hepatic peliosis. DMNA induced an appreciable number of vascular tumours in C3Hf females, particularly in the liver (see Table 1). Vascular tumours composed of capillaries alone were diagnosed as capillary hemangiomas (Fig. 2), those consisting of widely dilated vessels were classified as cavernous hemangiomas (Fig. 3). Mixed forms were also observed and classified according to the major component. Vascular tumours of small vessels which showed proliferation of atypical endothelial cells were classified as malignant hemangioendotheliomas. The vessel walls are covered by endothelial cells usually more than one cell layer thick (Figs. 4 and 5). Silver reticulin staining was

Table 3. Tumours and other lesions at other sites than liver in C3Hf male and female mice after DMNA administration

Group*	1	2	3	4	5	6	7	8	9
Sex	♂	♂	♂	♂	♀	♀	♀	♀	♀
Treatment	Control	Control	1 × DMNA	13 weeks DMNA	Control	Control	1 × DMNA	13 weeks DMNA	13 weeks DMNA
Number of lung tumour bearing animals	0	1(5%)	1(6%)	15(88%)	2(10%)	0	3(15%)	8(40%)	7(35%)
Mean number of lung tumours per tumour bearing animal	0	1·0	1·0	1·2	2·0	0	1·0	2·8	2·1
Number of animals with vascular tumours	0	0	0	0	0	1(5%)†	0	2(10%)†‡	3(15%)§
Number of animals with mesenteric disease	0	0	0	0	0	7(35%)‖	0	0	0
Other tumours	see¶	0	0	0	0	see**	0	see††	0

* For further details: see Table 1.
† Cavernous hemangioma of the ovary (1 ×).
‡ Hemangiosarcoma in the mesenterium (1 ×).
§ 2 Hemangiosarcomas of the soft tissues (one of them retroperitoneal); 1 cavernous hemangioma (in the abdominal fat).
‖ Involving not only mesenteric, but also inguinal, lumbar, renal, pyloric/pancreatic and superficial cervical lymph nodes.
¶ Lipoma (1 ×).
** Fibrosarcomas of the soft tissues (3 tumours in 3 animals), 2 mammary tumours in 2 animals (1 adenoacanthoma, 1 adenocarcinoma), lipoma (1 ×), granulosa cell tumour (1 ×), leukemia of reticulum cell type (2 ×).
†† Adrenal tumour (1 ×), leukemia of reticulum cell type (1 ×).

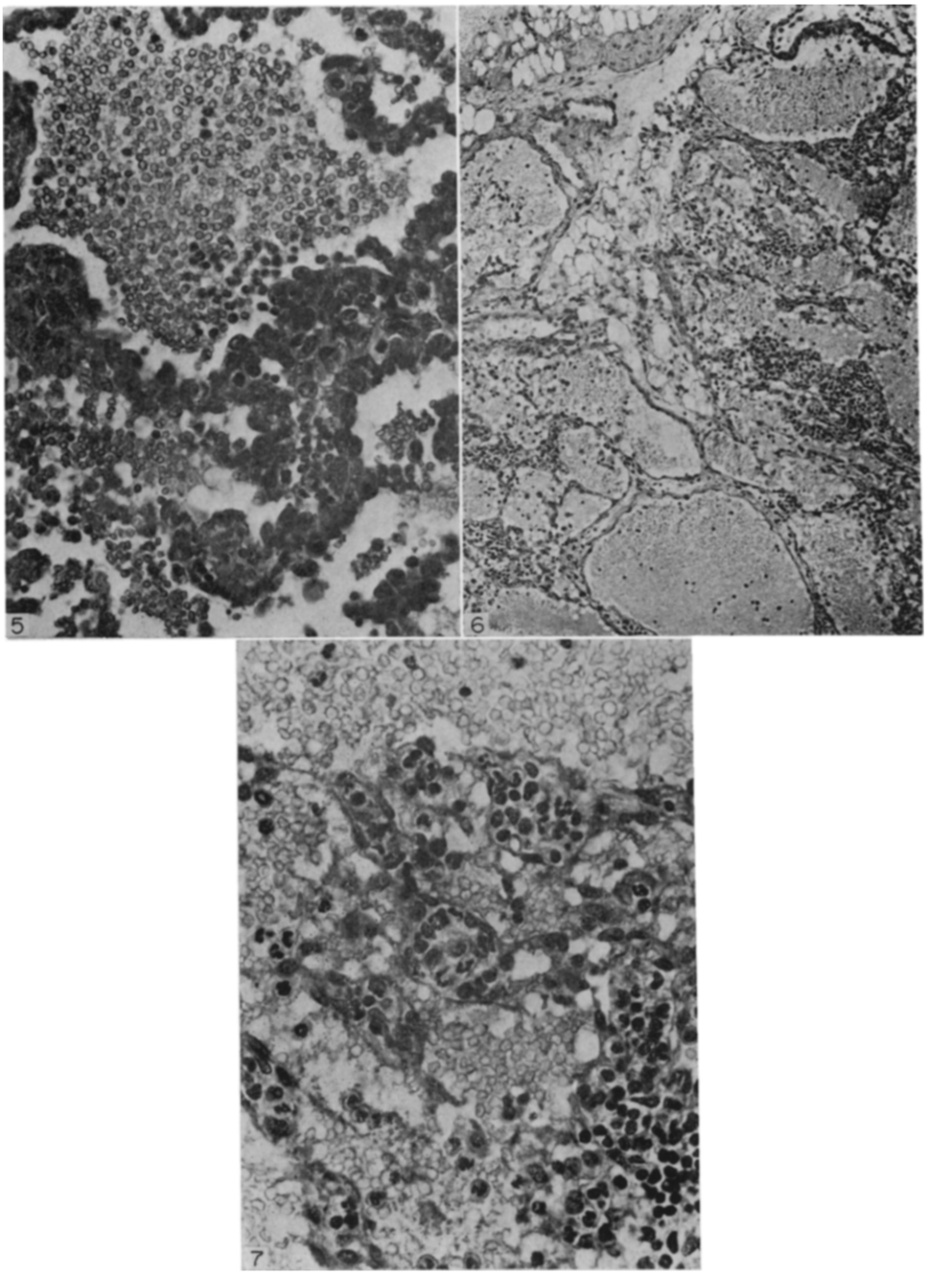


Fig. 5. *Detail of a hemangioendothelioma in the liver of a* 58 *weeks old C3Hf female mouse, treated for* 13 *weeks with DMNA in drinking water* (*Group* 8). *Several mitotic endothelial cells are visible.* × 250.

Fig. 6. *Lymph node of a* 99 *weeks old control C3Hf female mouse showing so-called mesenteric disease* (*see text*) (*Group* 6). *Note the presence of dilated sinuses filled with red blood cells.* × 120.

Fig. 7. *Another area of same section showing focal accumulation of granulocytes.* × 480.

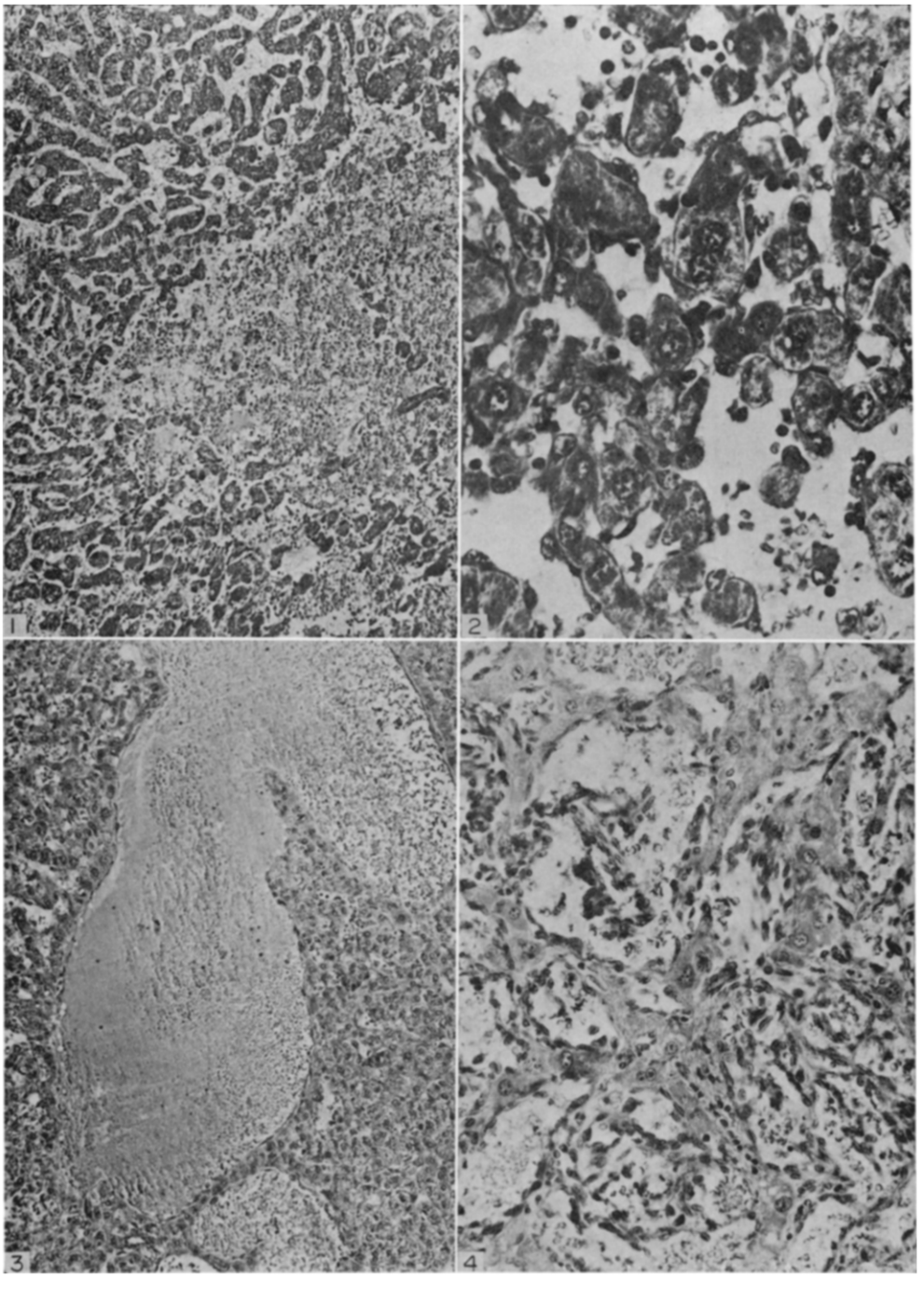


Fig. 1. *Liver of a* 51 *weeks old C3Hf female mouse, treated for* 13 *weeks with DMNA in drinking water. Peliosis is confined to the central and right parts whereas the left and upper parts show sinusoidal congestion (Group* 9). × 120.

Fig. 2. *Capillary hemangioma in the liver of a* 58 *weeks old C3Hf female mouse, treated for* 13 *weeks with DMNA in drinking water (Group* 8). × 480.

Fig. 3. *Cavernous hemangioma in the liver of a* 58 *weeks old C3Hf female mouse, treated for* 13 *weeks with DMNA in drinking water (Group* 8). *Blood-filled spaces are lined with endothelial cells.* × 120.

Fig. 4. *Hemangioendothelioma in the liver of a* 57 *weeks old C3Hf female mouse, treated for* 13 *weeks with DMNA in drinking water (Group* 9). *In some areas clusters of atypical endothelial cells are seen projecting into the lumen.* × 250.

[to face p. 132]

consistent with a proliferation of endothelial origin. From 40–60% of the animals in groups 8 and 9 showed a hemangioma and/or a hemangioendothelioma in this organ. The findings in treated C3Hf-females with their large number of blood-vessel tumours in the liver are contrasting to those in the males of the same strain, in which hepatomas were predominating.

Neoplastic lesions in other organs

In Table 3 the lesions in other organs than the liver are recorded. In C3Hf males, the administration of DMNA in drinking water during 13 weeks chiefly influenced the number of animals with lung tumours. In untreated C3Hf females at 58 weeks no tumours were found with the exception of some lung tumours. In untreated females of this strain at 99 weeks 7 out of 20 animals showed one or more tumours; one tumour was diagnosed as a cavernous hemangioma of the ovary. Other tumours found in this group were fibrosarcomas of the soft tissues (3×), mammary tumours (2×), lipoma (1×), granulosa cell tumour (1×), and leukemia (2×) involving respectively spleen (2×), liver (1×) and lymph nodes (1×).

Administration of DMNA during 13 weeks to female mice (groups 8 and 9) resulted in a significant increase in the number of lung adenomas and vascular tumours. Vascular tumours were found to be located in the ovary, the mesenterium, and the soft tissues. In C3Hf females treated for 13 weeks with DMNA a number of animals had lung lesions (group 8: 2 animals, group 9: 4 animals) with excessive bleeding in one or several lobes. Microscopically, blood filled alveoli were found together with cellular proliferation in the alveolar walls. One animal in group 8 showed leukemia of the reticulum cell type involving the spleen only, in another animal of this group an adrenal tumour was found but could not be further identified. Renal lesions were found in a substantial number of animals; because not all of these usually very small lesions were prepared for histological examination, meaningful figures can be given for groups 8 and 9 only, in which groups 3 (15%) and 1 (5%) animals with a renal adenoma were detected, respectively.

Non-neoplastic lesions in other organs

Non-neoplastic changes in a variety of lymph nodes were found in 99 weeks old control females (Figs. 6 and 7) and resembled so-called mesenteric disease in which the mesenterial lymph node is involved mainly [12]. In our experiment inguinal, lumbar, renal, mesenteric, pyloric/pancreatic and superficial cervical lymph nodes were involved. Mesenteric disease is not thought to possess a neoplastic character and was suggested [12] to be caused by venous congestion. Mesenteric disease was not seen in any of the female mice treated with DMNA.

Enlarged spleens were seen in a number of female C3Hf mice in group 6 (always in combination with mesenteric disease) and in groups 8 and 9 (in most cases in combination with vascular lesions); all enlarged spleens showed extreme extramedullary hemopoiesis.

DISCUSSION

Tumours of different cellular origin can be induced in one organ, depending on the experimental conditions. With regard to neoplastic changes in the liver, administration of DMNA to rodents has been found to result in hepatocellular, cholangiolar or vascular tumours in this organ [13]. In the mouse liver hepatocellular and vascular tumours may be induced by DMNA or diethylnitrosamine, the type of tumour depending on age [14], strain [15], carcinogen [15], the simultaneous administration of halogenated hydrocarbons [16], or the daily dosage of the carcinogen [17].

In the experiments reported in this paper, the type of tumour in the liver of C3Hf mice after DMNA administration was found to be influenced by the sex of the animals. Major tumour type in the male liver was the hepatoma whereas nearly all tumours in the female liver were of the vascular type. This phenomenon of sex-dependency might be related to the DMNA–N–demethylating activity of cells from which the tumours originate. Formaldehyde production by isolated liver microsomes of both C3Hf sexes was found to be very similar (unpublished results), indicating no or only minor differences in DMNA metabolism by parenchymal cells of male and female liver.

Histological and autoradiographical studies of mouse liver during the course of vascular tumourigenesis by DMNA showed both proliferation and ^{3}H-thymidine incorporation in sinusoidal endothelial cells [18]. This is of interest because extensive evidence indicates the importance of cellular proliferation in liver tumour induction (see [19] for review).

Because most animals in groups 8 and 9 (C3Hf females, 13 weeks DMNA) died between

30 and 50 weeks with extensive liver lesions, the possibility cannot be excluded that hepatoma formation in these animals was obscured by the development of lethal vascular liver lesions. The only hepatoma found in DMNA-treated females was located in an otherwise histologically normal liver. Preliminary experiments with lower DMNA dosages (13 weeks 5 ppm in drinking water) showed no hepatoma formation, suggesting that the tumour type in C3Hf-female liver is not dose-dependent. Apart from intrinsic differences in cellular sensitivities to tumour formation, a possible explanation for this sex-dependent phenomenon might be found in the widely diverging hormonal environments to which DMNA-treated cells are exposed. In this connection it might be relevant that a general difference in sensitivity between C3Hf males and females with regard to the formation of vascular tumours is indicated by the occurrence of extra-hepatic vascular tumours in the females (Table 3). Vascular lesions were found not only in 58 weeks old C3Hf females after a 13 weeks treatment with DMNA, but also in untreated ones at the age of 99 weeks. The high incidence of vascular tumours in the liver after oral DMNA administration (Groups 8 and 9) might be related to the route of carcinogen administration, since it is known that the number of liver tumours after oral administration of N,N–diamylnitrosamine is higher than after giving this compound subcutaneously [20]. Indirect evidence for a decisive role of some sex-dependent factor(s) in tumour development comes from the pronounced influence of operatively (e.g. castrated) or otherwise induced changes in the host hormonal status on the incidence of neoplastic lesions in the livers of mice [21] and rats [22, 23]. In female, but not in male Swiss mice the intraperitoneal injection of DMNA resulted in a substantial number of extrahepatic vascular tumours [24]. It was suggested [25] that the distribution of vascular tumours in humans is in accordance with the experimental situation, females being more susceptible than males (compare, however, [10]). Our results do not indicate a significant leukemogenic action of DMNA as was suggested by Otsuka and Kuwahara [11] in whose experiments however control groups were lacking.

Tumour formation after a single DMNA administration

Adult C3Hf males proved to be very sensitive to hepatoma formation, a single intraperitoneal injection of 7 mg DMNA/kg significantly increasing the hepatoma incidence at 58 weeks (Table 1, group 3). Formation of hepatomas in intact mice after one carcinogen injection has been reported for newborn or infant animals [26, 27] but not for adult ones. The exceptional sensitivity of adult C3Hf males to hepatoma development after a single injection (Table 1, group 3) might be related to the high sensitivity to spontaneous hepatoma formation (Table 1, group 2). The same treatment of 11–13 weeks old females (group 7; 1×7 mg DMNA/kg intraperitoneally) did not result in the formation of any tumour in the liver. This contrasting behaviour suggests the suitability of the C3Hf strain for investigating the role of carcinogen–nucleic acid interaction, host hormonal status and cellular proliferation in the development of hepatocellular and vascular tumours in the mouse liver after administration of dimethylnitrosamine. The presence of nitrosamines in the human environment [28] further stresses the need for clarifying the process of tumourigenesis by these compounds.

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REFERENCES

1. S. D. Vesselinovitch, The sex-dependent difference in the development of liver tumors in mice administered dimethylnitrosamine. *Cancer Res.* **29,** 1024 (1969).
2. S. C. Skoryna and D. R. Webster, Production of cirrhosis and liver tumors in male rats using high doses of 2-acetylaminofluorene. *Proc. Soc. exp. Biol. (N.Y.)* **78,** 62 (1951).
3. H. B. Andervont, H. G. Grady and J. E. Edwards, Induction of hepatic lesions, hepatomas, pulmonary tumors, and hemangioendotheliomas in mice with *o*-aminoazotoluene, *J. nat. Cancer Inst.* **3,** 131 (1942).

4. S. D. Vesselinovitch, N. Mihailovich, L. S. Lombard and G. N. Wogan, Aflatoxin B_1 carcinogenicity in infant mice. *Proc. Amer. Ass. Cancer Res.* **13,** Abstr. 195 (1972).
5. K. Stromberg and M. D. Reuber, Influence of age and sex on hepatic lesions induced by chemical carcinogens: ingestion of N-4-(4'-fluorobiphenyl)-acetamide by Buffalo strain rats, *J. nat. Cancer Inst.* **44,** 1047 (1970).
6. L. Den Engelse, P. A. J. Bentvelzen and P. Emmelot, Studies on lung tumours I. Methylation of deoxyribonucleic acid and tumour formation following administration of dimethylnitrosamine to mice. *Chem.-biol. Interactions* **1,** 395 (1969/1970).
7. H. B. Andervont and T. B. Dunn, Transplantation of spontaneous and induced hepatomas in inbred mice, *J. nat. Cancer Inst.* **13,** 455 (1952).
8. P. Emmelot and C. J. Bos, Studies on plasma membranes. X. A survey of enzyme activities displayed by plasma membranes isolated from mouse liver and three mouse hepatoma strains. *Int. J. Cancer* **4,** 723 (1969).
9. C. F. Hollander, G. A. Boorman and C. Zurcher, Classification of malignant tumours in rodents. *Proceedings of the 5th Perugia Quadrennial International Conference on Cancer*, 1973. (In press)
10. A. P. Stout and R. Lattes, Tumors of the soft tissues. AFIP, *Atlas of tumor pathology*, 2nd series, Fasc. **1,** pp. 67 and 145 (1967).
11. H. Otsuka and A. Kuwaraha, Hemangiomatous lesions of mice treated with nitrosodimethylamine. *Gann* **62,** 147 (1971).
12. T. B. Dunn, Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. *J. nat. Cancer Inst.* **14,** 1281 (1954).
13. IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Man, *IARC Monographs on the evaluation of the carcinogenic risk of chemicals to man.* **1,** 95 (1972).
14. B. Toth, P. N. Magee and P. Shubik, Carcinogenesis study with dimethylnitrosamine administered orally to adult and subcutaneously to newborn BALB/c mice. *Cancer Res.* **24,** 1712 (1964).
15. N. K. Clapp, R. L. Tyndall and J. A. Otten, Differences in tumor types and organ susceptibility in BALB/c and RF mice following dimethylnitrosamine. *Cancer Res.* **31,** 196 (1971).
16. W. Kunz, G. Schaude and C. Thomas, Die Beeinflussung der Nitrosamin-carcinogenese durch Phenobarbital und Halogenkohlenwasserstoffe. Z. *Krebsforsch.* **72,** 291 (1969).
17. N. K. Clapp and R. E. Toya sr., Effect of cumulative dose and dose rate on dimethylnitrosamine oncogenesis in RF mice. *J. nat. Cancer Inst.* **45,** 495 (1970).
18. S. Takayama, The histological and autoradiographical studies of mouse liver during the course of carcinogenesis by dimethylnitrosamine. *Z. Krebsforsch.* **71,** 246 (1968).
19. G. P. Warwick, Effect of the cell cycle on carcinogenesis. *Fed. Proc.* **30,** 1760 (1971)
20. H. Druckrey and R. Preussmann, Erzeugung von Lungenkrebs durch subcutane Injektion von N,N-diamylnitrosamin an Ratten. *Naturwiss.* **5,** 111 (1962).
21. H. B. Andervont and T. B. Dunn, Effect of castration and sex hormones on the induction of tumors in mice with *o*-aminoazotoluene. *J. nat. Cancer Inst.* **7,** 455 (1947).
22. M. D. Reuber, Effect of growth hormone on hepatic carcinogenesis and cirrhosis in A×C rats given N-2-fluorenyldiacetamide. *Cancer Res.* **28,** 2177 (1968).
23. C. M. Goodall, Endocrine factors as determinants of the susceptibility of the liver to carcinogenic agents. *N.Z. med. J.* **67,** 32 (1968).
24. A. Cardesa, P. Pour, J. Althoff and U. Mohr, Vascular tumors in female Swiss mice after intraperitoneal injection of dimethylnitrosamine, *J. nat. Cancer Inst.* **51,** 201 (1973).

25. R. W. EVANS, *Histological Appearances of Tumours*, pp. 91–92, E. and S. Livingstone: Edinburgh (1966).

26. B. TOTH, A critical review of experiments in chemical carcinogenesis using newborn animals. *Cancer Res.* **28,** 727 (1968).

27. G. DELLA PORTA and B. TERRACINI, Chemical carcinogenesis in infant animals. *Prog. exp. Tumor Res.* **11,** 334 (1969).

28. P. BOGOVSKI, R. PREUSSMANN and E. A. WALKER (Editors), N-Nitrosocompounds analysis and formation, *IARC Scient. Publ. No.* **3** (1972).

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The Uptake, Distribution and Anti-Tumor Activity of Bleomycin in Gliomas in the Mouse

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Abstract—*The uptake and distribution of ^{14}C-Bleomycin-A_2 in mice with methylcholanthrene induced gliomas was investigated by radioactive assay and bioassay, and also the inhibitory effect of Bleomycin on the growth of subcutaneously transplanted gliomas in mice was studied. Evidence has been presented:* (1) *A relatively large amount of Bleomycin may be taken up selectively into gliomas by intraveneous administration and about two thirds of Bleomycin in the glioma may remain in so-called active form.* (2) *Large doses of Bleomycin may have significant anti-tumor activity against gliomas.*

BLEOMYCIN (BLM), discovered by Umezawa *et al.* [1, 2], is a new cytostatic antibiotic complex produced by a strain of *Streptomyces verticillus* and was found by Ichikawa *et al.* [3, 4] to be effective against human squamous cell carcinoma. These authors observed relatively high concentrations of BLM in the skin after subcutaneous injection in mice, and suggested that the therapeutic effect on squamous cell carcinoma might be related to the high concentration of BLM in the skin [5–8].

It has been suggested that BLM might be effective against gliomas, since the central nervous system, like skin, is an ectodermal organ. The present study has been designed to investigate the distribution of BLM in mice with methylcholanthrene-induced gliomas and to study the effect of BLM on the growth of gliomas transplanted subcutaneously in mice.

MATERIAL AND METHODS

A. Uptake of BLM by murine gliomas

(1) *Induction of brain tumor.* Brain tumor was induced in male ddN mice by the technique of Zimmerman and Arnold [9]. A pellet of 20-methylcholanthrene, pure fused crystal about 1 mg, was implanted in the right parietal region of 5-week-old animals weighing approximately 15 g. The animals were observed carefully and received a single intravenous injection of ^{14}C-Bleomycin-A_2 when they showed malaise and neurological signs, i.e. paralysis, circling movement, ataxia or convulsion, or bulging of the skull, and were suspected of having a brain tumor.

(2) *Injection of BLM.* ^{14}C-labelled Bleomycin-A_2 (7·52 μCi/mg) (Bleomycin-A_2: the main component of BLM mixture, and the chemical structure and the site of ^{14}C-labelling is shown in Fig. 1) was obtained from Nihon Kayaku Co., Tokyo, and was prepared 2 μCi/mg by addition of cold Bleomycin-A_2. The mouse suspected of having a brain tumor was given a single intravenous injection of ^{14}C-Bleomycin-A_2, 0·1 mg/g body weight into a tail vein.

(3) *Measurement of radioactivity.* The animals were bled to death by cardiac puncture two hr after the injection. The brain, with the brain tumor, liver, kidney, lung and skin of the back were removed immediately after death, cut into pieces, stored in closed vials and weighed accurately, as soon as possible, in a cold room. The tissue radioactivity was measured by the method based on Dulcino *et al.* [10] and the plasma radioactivity, by the method of Meade

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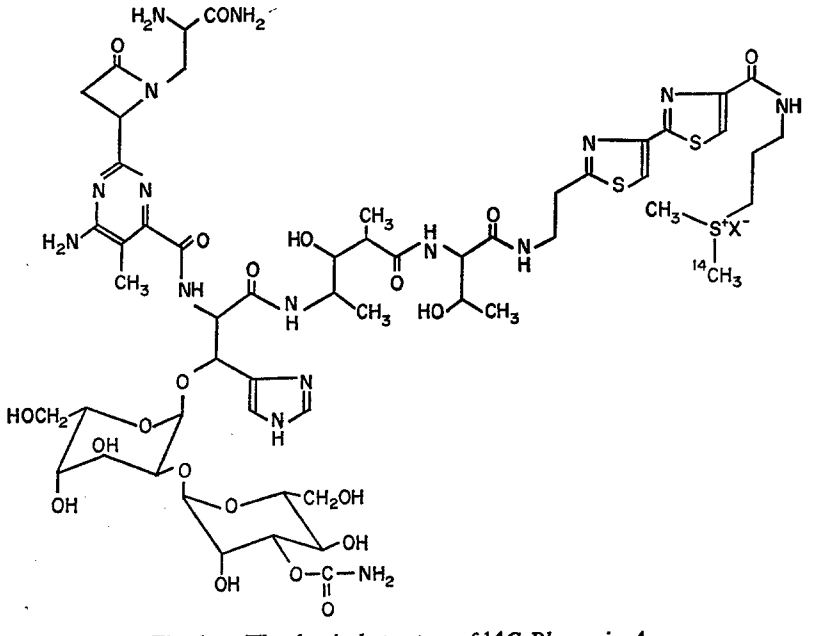


Fig. 1. *The chemical structure of* ^{14}*C-Bleomycin-A*$_2$.

et al. [11]. The scintillating mixture was stored for 24 hr and the counts were made with the liquid scintillation counter (Nuclear Chicago, Mark I). The counting efficiency was 20–40%.

B. BLM concentrations in gliomas

In order to know the ratio of active form of BLM to total BLM taken up by the glioma, the distribution of ^{14}C-Bleomycin-A$_2$ in subcutaneously transplanted gliomas was measured by radioactive counting as well as bioassay.

(1) *Experimental glioma model.* As the methylcholanthrene-induced gliomas *in situ* were too small to be employed for the determination of antibacterial activity, subcutaneously transplanted tumors derived from methylcholanthrene-induced gliomas were used. A piece of glioma tissue (about 30 mg of weight) was transplanted subcutaneously in the lower abdominal quadrant of male ddN mice weighing approximately 20 g. Three weeks after the transplantation, the tumors grew to about 1·5 cm in diameter and the animals were given an intravenous injection of ^{14}C-Bleomycin-A$_2$.

The methods of the injection of ^{14}C-Bleomycin-A$_2$ and the measurement of it by radioactivity were the same as those in the above Experiment A.

(2) *Measurement of BLM by antibacterial bioassay.* The paper disc plate method [12, 13] using *B. subtilis* spores was employed for the determination of BLM concentration by its antibacterial activity. *Bacillus subtilis* PCI strain was inoculated on a nutrient agar slant, cultured for one week at 37°C, and two ase of the spores were suspended in one ml of sterile distilled water and inactivated for 30 min at 65°C. The spore suspension was washed three to five times with sterile distilled water, centrifuged for 10 min at 2000 rev/min, inactivated for 30 min at 65°C, and diluted 200-fold with sterile distilled water. The resultant spore suspension was inoculated on nutrient agar medium (polypeptone 10·0, meat extract 5·0, agar 20·0, aq. dest. 1000 ml, and adjusted to pH 6·8) to the concentration of 0·15–0·3%. Five ml of the inoculated medium was poured evenly into flat bottomed Petri dishes, allowed to harden and kept in an ice box.

The pieces of tumor and organs were homogenized by a teflon homogenizer (Takashima Co. Ltd.: median type) in physiological saline solution, centrifuged, and the supernatants were used as for assay. 0·05 ml of the solution for assay was put on a paper disc (Toyo Paper Disc for Antibiotic Examination (Thick): Size 8 mm dia, Toyo Roshi Co. Ltd.) and this was placed on the surface of the inoculated agar plate. Three discs were used per test sample. After 2 hr of pre-incubation at 5°C in refrigerator for diffusion and enzymatic reaction, followed by 16 hr of incubation for cultivation at 37°C, the diameters of growth-inhibition zone were measured with calipers. The mean value of three diameters of inhibition zones was plotted on semi-log paper against the log concentration of standard BLM solution prepared by diluting cold Bleomycin-A$_2$ to 1–100 mcg/ml, with

mixture of M/15 Sorensen phosphate buffer, pH 7·2 and physiological saline. The concentration in the test sample was estimated by comparison with the standard curve. The minimum assayable concentration was 0·01 mcg/ml.

C. Inhibitory effect of BLM on the growth of gliomas

(1) *Experimental glioma model.* The glioma originally induced by methylcholanthrene in a mouse and maintained for 18–20 generations by serial subcutaneous transplantation every three weeks was used for the experiment. The tumor was histologically stable and its growth was constant.

Three weeks after the subcuntaneous transplantation in the right lower abdominal quadrant in male ddN mice weighing about 20 g, the tumor had grown to 1·0–1·5 cm dia and the treatment was started.

The mice with a transplanted tumor were separated into 6 groups of 10 mice each. BLM mixture at a single dose level of 1·5, 5, 15, 50 and 100 mg/kg body weight was injected intraperitoneally 5 times on alternate days, physiological saline solution was used as the control. Food and water were supplied *ad libitum* and every 5 days after the first injection the tumor sizes were measured by caliper and the body weight recorded.

RESULTS

A. Uptake of BLM by mouse gliomas

Five animals received a single injection of ^{14}C-Bleomycin-A_2, and in three of them autopsy showed an infiltrating primary tumor successfully induced in the brain (Photo 1). The histological examination of the tumors confirmed them to be malignant gliomas (Photo 2). The average induction time of the tumors was 7·1 months.

The radioactivity in the glioma *in situ* and various organs two hours after intravenous injection of ^{14}C-Bleomycin-A_2 are given in Fig. 2. The radioactivity indicated high concentrations of BLM in kidney, liver, lung and glioma. The radioactivity in the brain was significantly lower compared with that in glioma; the ratio of concentration in glioma to that in the brain was 3·0.

B. Comparison of BLM concentrations in glioma as measured by bioassay and radioactivity

The average distribution of ^{14}C-Bleomycin-A_2 measured radioactively and by bioassay in five mice with subcutaneously transplanted glioma is shown in Fig. 3.

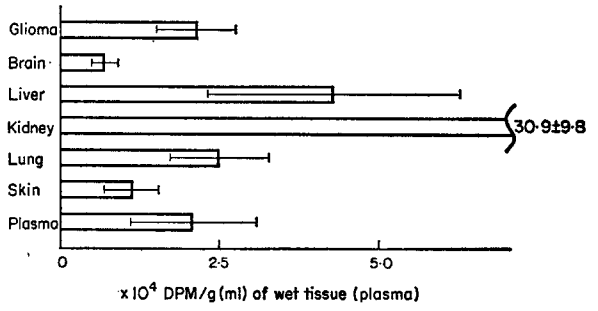


Fig. 2. Distribution of ^{14}C-Bleomycin-A_2 in methylcholanthrene-induced primary gliomas and various organs at 2 hr after intravenous injection of ^{14}C-Bleomycin-A_2, 0·2 μCi/0·1 mg/g body weight, in mice.

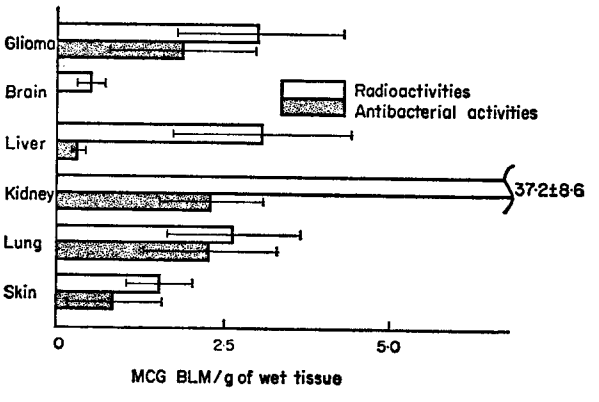


Fig. 3. Distribution of ^{14}C-Bleomycin-A_2 in subcutaneously transplanted glioma and organs determined by radioactivity and antibacterial activity 2 hr after intravenous injection of ^{14}C-Bleomycin-A_2, 0·2 μCi/0·1 mg/g body weight, in mice.

Concentration of ^{14}C-Bleomycin-A_2 in the organs as determined by radioactivity showed kidney, liver, lung and skin was the order of decreasing concentration. On the other hand, the concentration measured by bioassay showed high values in the kidney, and lung, followed by the skin. Although the concentration in the brain was very low by radioactive assay and was not detected by bioassay, the concentration in glioma was fairly high by radioactive and by bioassay. The ratios of concentration determined by bioassay to the radioactivity in the glioma were high as those in lung and skin, and the mean value of the ratio in the glioma was 0·62. Photograph 3 shows the inhibitory zones of growth of *B. subtilis* on an agar plate by ^{14}C-Bleomycin-A_2 in the glioma, brain tissue and plasma in a mouse.

C. Inhibitory effect of BLM on growth of glioma

The effects of various doses of BLM (1·5–100 mg/kg × 5) on the tumor growth and body weight changes in mice with subcutaneously transplanted gliomas are shown in Figs. 3 and 4.



Photo. 1. *Coronal section of a mouse brain with methyl-cholanthrene-induced malignant glioma.*

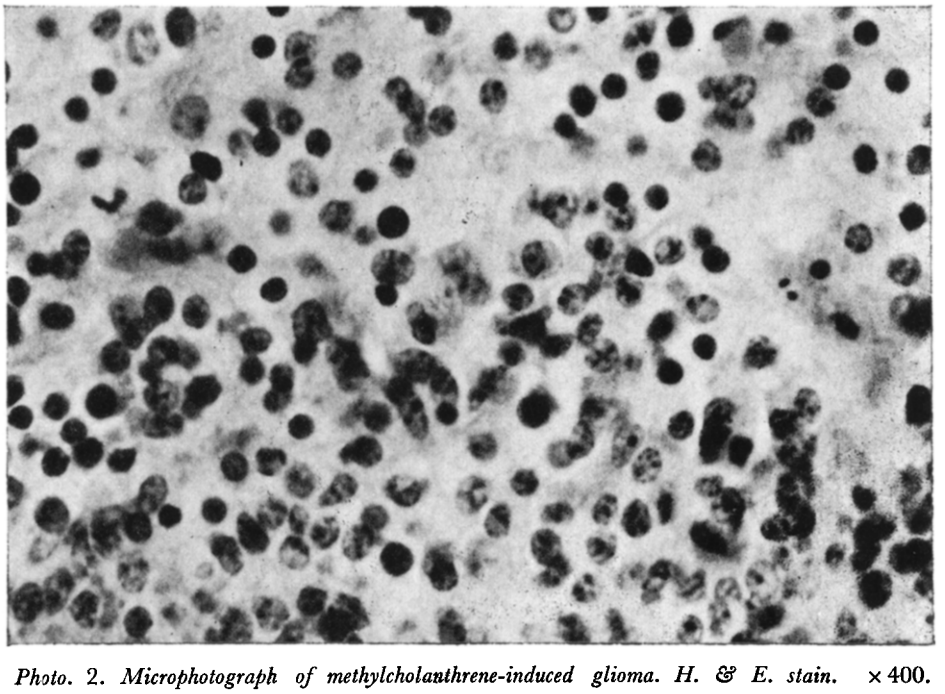


Photo. 2. *Microphotograph of methylcholanthrene-induced glioma. H. & E. stain.* ×400.

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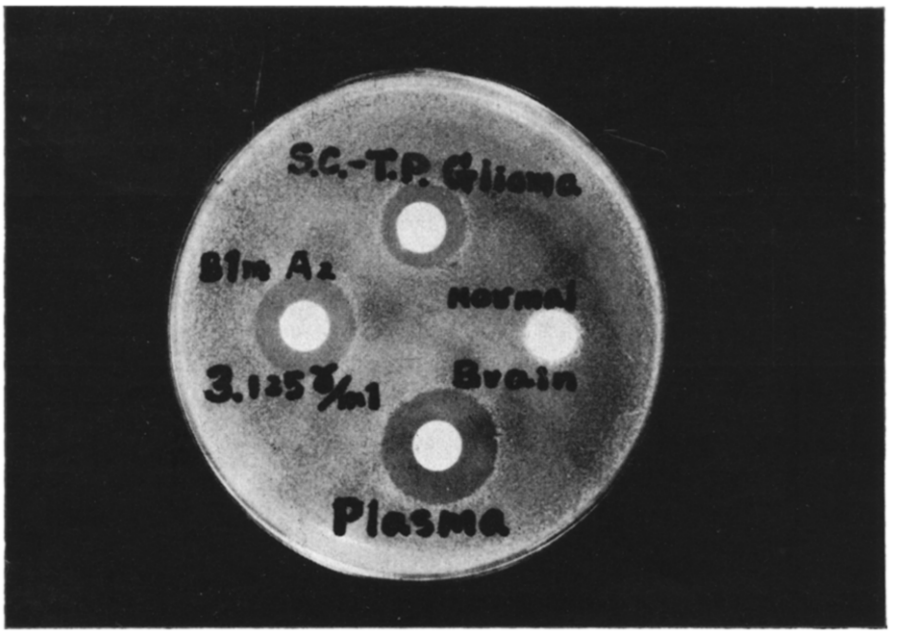


Photo. 3. *Inhibitory zones of growth of* B. subtilis *on an agar plate by* ^{14}*C-Bleomycin-A*$_2$ *in the glioma, brain tissue and plasma in a mouse.*

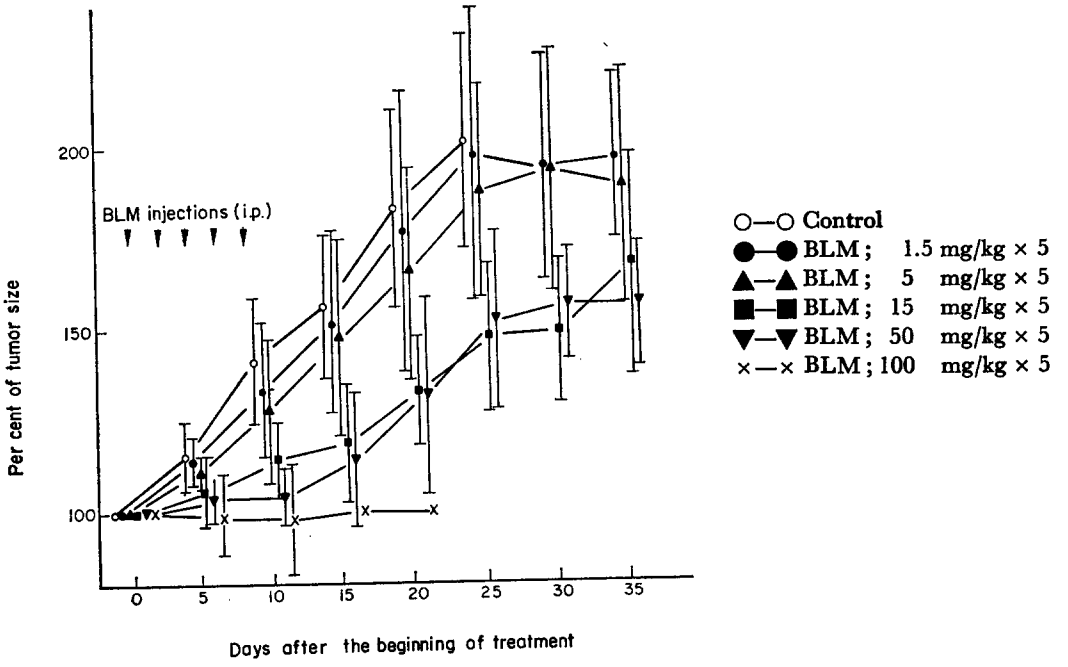


Fig. 4. Effect of Bleomycin (BLM) on the growth of subcutaneously transplanted glioma in mice. Tumor size immediately before treatment in each animal is taken as 100%. *Each group contained* 10 *animals.*

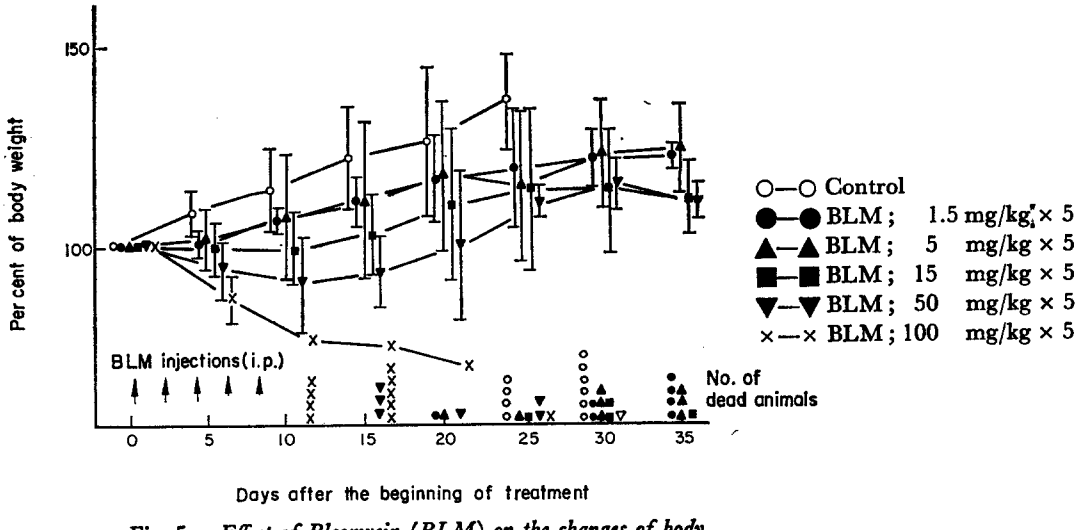


Fig. 5. Effect of Bleomycin (BLM) on the changes of body weight in mice with subcutaneously transplanted gliomas. Body weight immediately before treatment on each animal is taken as 100%. *Each group contained* 10 *animals.*

In the control animals, the tumor size became progressively larger and all animals died within 20–30 days after the beginning of the treatment (average survival time; $23{\cdot}0 \pm 2{\cdot}4$ days) from the start of treatment.

In the animals treated with BLM in a single dose of more than 15 mg/kg, the inhibitory effect of BLM on tumor growth was significant (Wilcoxon Rank Test; $P < 0{\cdot}01$), and even with the lower dose level of 5 mg/kg, a slightly inhibitory tendency in tumor growth was observed ($P < 0{\cdot}05$).

On the other hand, the rise of body weight was slowed to some degree in the animals treated with BLM compared with the controls, especially animals given BLM in a single dose

level larger than 50 mg/kg who lost weight daily and died soon after the initiation of BLM treatment.

The average survival times of the groups treated with BLM in a single dose of 1·5, 5, 15, 50 or 100 mg/kg were 29·0 ± 5·8, 28·5 ± 8·4, 37·0 ± 8·1, 26·0 ± 16·9 and 9·0 ± 4·4 days, respectively. These results indicate that the treatment with BLM in a single dose of 15 mg/kg, 5 times on alternate days, significantly prolongs the survival time of the mice with subcutaneously transplanted gliomas ($P < 0·01$).

DISCUSSION

Umezawa and his colleagues, who discovered BLM, investigated the distribution of Bleomycins in organs by radioactive assay and antibacterial assay in mice. They suggested that the therapeutic effect on squamous cell carcinoma and the toxic side effects which appear in the skin and lung were related to the high concentrations and the high ratios of the active form of BLM determined by bioassay, to the total BLM, as determined by radioactivity, in these organs [5–8].

In the present study, BLM given intravenously had a relatively high uptake by the glioma, though little went into the brain tissue. About two thirds of BLM taken up into the glioma remained in so-called active form as shown by its antibacterial activity.

Unlike previous experiments on the uptake of drugs by brain tumors using intracerebrally transplanted glial or non-glial tumors as models, chemical carcinogen-induced gliomas *in situ* were employed in this study, for it was very similar both macroscopically and microscopically to human malignant gliomas and well suited as a model for glioma.

Although the concentration of BLM in the kidney was extremely high in the present experiments, it is uncertain whether the value obtained in the kidney demonstrates the real concentration in the tissue, for the kidney was contaminated by urine which may have contained a high concentration of BLM [5–8]. A high concentration was also shown in the liver by radioactive assay, but the amount of BLM in the active form detected by bioassay was very low. The considerable variation in the concentration of the skin, particularly as determined by bioassay, may be due to the method of homogenization.

Subcutaneously transplanted glioma was used for the determination of the concentration ratio of active BLM shown by antibacterial activity to total BLM, for it was thought the ratio might be similar to that in the primary glioma.

There have only been a few reports on the distribution of BLM in brain tumors. Kanno *et al.* [14] investigated the distribution of ^{3}H-Bleomycin-A_2 in a mouse with methylcholanthrene induced meningosarcoma and observed the high concentration in the tumor, but the tumor model they used was non-glial and extracerebral, and the results could be extrapolated to suggest there would be an uptake of BLM by malignant gliomas in man. Takeuchi *et al.* [15, 16] conducted an extensive study on clinical application of BLM for the treatment of brain tumors. They investigated the concentration of BLM in brain tumor tissue in patients by bioassay, and detected 0·9 mcg/g of BLM in glioma tissue only in one out of ten with glioma investigated.

The cytostatic effect of BLM on brain tumors was first demonstrated by Kanno *et al.* [17]. They observed that BLM inhibited the growth curves of cultured human gliomatous and meningiomatous cells. Our findings on the inhibitory effect of BLM on the growth of subcutaneously transplanted gliomas also indicate that BLM has anti-tumor activity against gliomas, but it also appeared that it may be necessary to give a very large dose of BLM, which may cause toxic side effects, in order to obtain any significant effect against gliomas by routine systemic administration.

Based on these experimental findings, patients with malignant brain tumors have been treated with systemic, intracarotid or intrathecal chemotherapy with BLM with or without radiotherapy in our clinic. However, the series as yet is too small for any conclusions to be drawn.

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REFERENCES

1. H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, New antibiotics, Bleomycin A and B. *J. Antibiotics* **19A,** 200–209 (1966).
2. H. Umezawa, Y. Suhara, T. Takita and K. Maeda, Purification of bleomycins. *J. Antibiotics* **19A,** 210–215 (1966).
3. T. Ichikawa, Treatment of squamous cell carcinoma with a new antitumor antibiotic, Bleomycin (BLM). *Jap. Med. J.* **2269,** 37–40 (1967).
4. T. Ichikawa, I. Nakano, I. Hirokawa and M. Murata, On the treatment of skin tumors, including penile cancer, with Bleomycin. *Chemotherapy* **16,** 882–903 (1968).
5. T. Ichikawa, A. Matsuda, K. Miyamoto, M. Tsubosaki, T. Kaihara, K. Sakamoto and H. Umezawa, Biological studies on Bleomycin A. *J. Antibiotics* **20A,** 149–155 (1967).
6. H. Umezawa, M. Ishizuka, K. Maeda and T. Takeuchi, Studies on Bleomycin. *Cancer* **20,** 891–895 (1967).
7. H. Umezawa, M. Ishizuka, K. Kimura, J. Iwanaga and T. Takeuchi, Biological studies on individual bleomycins. *J. Antibiotics* **21A,** 592–602 (1968).
8. H. Umezawa, M. Ishizuka, S. Hori, H. Chimura, T. Takeuchi and T. Komai, The distribution of ^{3}H-Bleomycin in mouse tissue. *J. Antibiotics* **21A,** 638–642 (1968).
9. H. M. Zimmerman and H. Arnold, Experimental brain tumors. 1. Tumors produced with methylcholanthrene. *Cancer Res.* **1,** 919–938 (1941).
10. J. Dulcino, R. Bosco, W. G. Verly and J. R. Maisin, Assay of tritium and carbon-14 in animal tissues by liquid scintillation. *Clin. Chim. Acta,* **8,** 58–65 (1963).
11. R. C. Meade and R. A. Stiglitz, Improved solvent systems for liquid scintillation counting of body fluids and tissues. *Int. J. appl. Rad. Isotopes* **13,** 11–14 (1962).
12. H. Ericsson, The paper disc method for determination of bacterial sensitivity to antibiotics. *Scand. J. Clin. Lab. Invest.* **12**(4), 408–413 (1960).
13. Y. Kanazawa, Single disc method for minimum inhibitory concentration (MIC) determination. *J. Antibiotics* **19A,** 175–189 (1966).
14. T. Kanno, T. Kudo, T. Nakazawa, T. Takeuchi and H. Umezawa, Bleomycin as an anti-brain tumor antibiotic. Part II. The distribution of ^{3}H-Bleomycin-A_2(-Cu) in the experimental brain tumor of mouse. *Clin. Neurol.* **10,** 501–506 (1970).
15. K. Takeuchi, M. Atsuji, T. Masui, S. Sueyoshi, M. Hara, M. Okada, R. Ogiwara and M. Oyama, Chemotherapy of brain tumor. Effect of Bleomycin. *Brain Nerve* **24,** 439–444 (1972).
16. K. Takeuchi, Determination of Bleomycin concentration in brain tumor tissue by bioassay. Committee of Clinical Research of Bleomycin, Proceedings of the 3rd Brain Tumor Meeting, Tokyo, March, 1971.
17. T. Kanno, T. Kudo and T. Nakazawa, Anti-brain tumor activity of Bleomycin. *Clin. Neurol.* **10,** 409–414 (1970).

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Chromosome Studies on 14 Near-Diploid Carcinomas of the Ovary

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Abstract—*Chromosome analysis of 26 samples from 14 near-diploid carcinomas of the ovary indicated that in 13 carcinomas a single aneuploid clone was present. In one, however, two regions of the primary had different karyotypes. The data suggest that ovarian carcinomas, including those in which both ovaries are involved, are commonly but not invariably monoclonal. The significance of a substantial proportion of diploid cells in one ovary from a bilateral case is discussed.*

In continuation of a previous study [1] on the chromosomes and DNA content of ovarian carcinomas which showed that the tumours fell into two groups having low (near-diploid) and high (near-triploid or near-tetraploid) modes respectively, we have made detailed karyotype analyses on 14 carcinomas of the ovary with near-diploid modes. In particular, evidence has been sought for a clonal relationship between the cells in each tumour. Where possible, samples from different regions of the tumour were compared.

MATERIAL AND METHODS

Table 1 gives brief details of the cases, six of which were included in the previous report [1]. Direct chromosome preparations from solid tumour tissue or malignant ascites, made as previously described [2], were stained in orcein. Attempts to produce chromosome banding by quinacrine mustard and the ASG technique of Sumner *et al.* [3] were unsuccessful with one exception (Case No. 4), presumably due to the age of the material.

RESULTS AND COMMENT

In Table 2, a summary of the karyotype analyses is given. It was evident that within each tumour sample the aneuploid metaphases had similar karyotypes and belonged to the same clone. Furthermore, except for the two regions of the primary tumour of Case No. 11 which had different karyotypes, different samples from the same case also showed close similarities. In particular, tumour from both ovaries of five cases was studied, and in each it was obvious that the aneuploid population of malignant cells in the two ovaries belonged to the same clone. Tumour from a peritoneal metastasis and ascitic fluid obtained from Case No. 9 eight months after removal of the bilateral ovarian tumours showed small differences (with a slightly higher chromosome number) as well as similarities, compared with the ovarian tumours.

Tumour from one of the ovaries of Case No. 1 was noteworthy in containing a substantial proportion of diploid metaphases (including 8 out of 16 analysed or counted). The chromosomes of the diploid metaphases had a generally better morphology and were less contracted than those of the aneuploid metaphases (a diploid and an aneuploid metaphase from this case have been illustrated elsewhere [4]) .In keeping with our general experience, the good morphology of the diploid metaphases is consistent with their being normal cells. However, an examination of histological sections showed that relatively few mitoses were present among the stromal cells. It is suggested, therefore, that the diploid cells were tumour cells, but that they were non-malignant and were remnants of a

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Table 1. *Summary of histopathological and clinical data, and modal chromosome numbers, of the* 14 *ovarian carcinomas. All the material was untreated except the peritoneal metastasis and ascitic fluid from Case No.* 9 *which were obtained after Chlorambucil therapy*

Case Number. In brackets: Case Number in previous study (1)	Age	Histopathological type. In brackets; Broders grade	Regions studied	Number of metaphases counted/ analysed	Modal chromosome number	Time in months to last appointment or death. A = alive D = died
1	45	Serous papillary cystadenocarcinoma (3)	1 left ovary	11/5	38	D 11
			2 Right ovary	16/5*	38	
			3 Omental deposit	10/4	38	
			4 Ascitic fluid	18/8	38	
2 (1)	53	Serous papillary adenocarcinoma (3)	Right ovary	15/4	39	D 15
3	60	Endometrioid carcinoma (3)	1 Right ovary	18/18	40	D 12
			2 Left ovary	10/10	40	
4	55	Endometrioid carcinoma (3)	Mass near right ovary	66/30	40	D 19
5 (4)	61	Mesonephroma (3)	Left ovary	18/12	40–41	D 5
6	86	Endometrioid	1 Left ovary, region 1	11/5	40–41	D 22
(3)		carcinoma (2)	2 Left ovary, region 2	8/7	41	
			3 Ascitic fluid	55/11	41–42	
7	47	Serous papillary	1 Right ovary	15/11	41–42	D 56
(2)		cystadenocarcinoma (3)	2 Left ovary	19/5	41	
8	52	Mixed mesodermal tumour (3)	1 Right ovary	13/4	40–41	D 12
			2 Left ovary	45/22	42	
9	52	Serous papillary	1 Right ovary	56/13	42	D 11
(5)		cystadenocarcinoma (2)	2 Left ovary	47/20	41	
			8 months later:			
			3 Peritoneal metastasis	38/13	42–44	
			4 Ascitic fluid	27/8	43–44	
10	48	Mucinous cystadenocarcinoma (2)	Right ovary	41/9	41–42	A 25
11	52	Endometrioid	1 Right ovary, region 1	29/16	44–46	D 20
(6)		carcinoma (2)	2 Right ovary, region 2	20/13	41–42	
12	60	Endometrioid carcinoma (2)	Right ovary	96/22	46–47	D 25
13	49	Partly endometrioid carcinoma and partly serous papillary cystadenocarcinoma (a grade 1 carcinoma was also present) (3)	Tumour in pelvis	59/18	47–48	D 5
14	35	Probably a malignant teratoma rather than a mixed mesodermal tumour in view of diversity of tissues	Right ovary	40/9	48–49	A 35

* Five aneuploid cells were analysed; diploid cells were also present (see text).

Table 2. Summary of karyotype analyses giving a single most commonly occurring karyotype from each tumour, except Case No. 11 where a karyotype from each of the two regions is shown. Based on orcein preparations

Case Number		Chromosome group: A 1	A 2	A 3	B	C	D	E 16	E 17–18	F	G	Marker chromosomes	Total
1		1	1	2	2	15	5	2	3	2	4	1	38
2		2	2	2	2	13	3	2	3	4	3	3	39
3		2	1	2	2	13	2	2	4	4	3	5	40
4		2	1	2	1	10	1	5		4	2	12†	40
5		2	2	1	3	14	4	2	4	4	3	2	41
6		2	1	2	2	13	4	7		4	3	3	41
7		1	1	2	2	16	2	2	4	3	4	4	41
8		3	2	3	3	13	2	3	3	4	3	2	41
9		1	2	2	3	14	4	2	3	3	3	5	42
10		2	2	5	4	13	3	2	1	5	3	2†	42
11	Region 1	2	2	2	2	18	4	4		3	4	2	43
	Region 2	1	2	1	3	18	3	5		3	3	3	42
12		0	2	5	3	17	4	4	3	3	4	1	46
13		2	2	4	4	16	6	2	3	4	4	0	47
14		2	2	2	4	17	8	2	3	4	4	0	48
Average number expressed as % of expected number		91·0	91·0	134·7	72·9	100·0	66·9	97·2		98·4	91·0	—	
								123·5*	84·0*				

* Estimated from the data on 11 tumours where these two subgroups were distinguished.
† Including one ring chromosome.

population present in the lesion when it was at a preinvasive stage. If this interpretation is correct, the ovary containing the diploid cells was the primary site; evidently only the aneuploid cells had metastasised since, with occasional exceptions which may have represented normal cells in mitosis, only aneuploid cells were found in the other tumour regions.

The analyses based on orcein preparations (Table 2) show a tendency for a reduction in the proportion of chromosomes in groups B, D and G which has been noted in tumours at other sites [5]. Markers, including one or more which were at least as large as the group A chromosomes, were present in each of the 11 hypodiploid tumours, whereas two of the three hyperdiploid tumours showed no obvious markers; large markers may be constantly present in hypodiploid tumours but markers of any size (at least in orcein preparations) are often lacking in hyperdiploid tumours, irrespective of their site or histopathological type [4].

The orcein preparations of Case No. 4 indicated that at least 12 markers were present.* These included two giant metacentrics whose morphology in the orcein preparations was similar but whose banding patterns differed. The banding patterns also suggested that the second "A3" was abnormal but in general were not sufficiently detailed to enable the status of the ? normal chromosomes to be verified or the origin of the abnormal chromosomes to be determined.

The data thus confirm the clonal nature of the malignant cell populations in carcinoma of the ovary and suggest that the tumours as a whole are commonly monoclonal, including most of the cases where both ovaries are involved. However, more than one clone may sometimes be present, as in Case No. 11 where two regions of the primary tumour yielded different karyotypes (a giant metacentric chromosome about 1·8 times the length of the No. 1 chromosomes was present in one region but not the other).

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*Photographs of orcein-stained and banded chromosomes from this tumour are available on request from the senior author.

REFERENCES

1. N. B. Atkin, Modal DNA value and chromosome number in ovarian neoplasia. A clinical and histopathologic assessment. *Cancer* **27,** 1064 (1971).
2. N. B. Atkin and M. C. Baker, Chromosome abnormalities as primary events in human malignant disease: evidence from marker chromosomes. *J. nat. Cancer Inst.* **36,** 539 (1966).
3. A. T. Sumner, H. J. Evans and R. A. Buckland, New technique for distinguishing between human chromosomes. *Nature New Biol.* **232,** 31 (1971).
4. N. B. Atkin, Chromosomes in human malignant tumors: a review and assessment. In *Chromosomes and Cancer* (Edited by J. German). John Wiley, New York (In press).
5. N. B. Atkin and M. C. Baker, Possible differences between the karyotypes of preinvasive lesions and malignant tumours. *Brit. J. Cancer* **23,** 329 (1969).

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Effect of B_6 Deficiency on Hepatoma 7794A Growth Rate: Activities of Tyrosine Transaminase and Serine Dehydratase Before and After Induction by Hydrocortisone*

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Abstract—*Vitamin B_6 is required for normal growth and functions as a coenzyme of many enzymes. The effect of the absence of this growth factor on* (a) *the growth of the well differentiated Morris hepatoma #7794A,* (b) *the activity of two vitamin B_6-requiring enzymes, i.e. tyrosine transaminase (TT) and serine dehydratase (SD), and* (c) *the induction of these by hydrocortisone in normal liver, host liver, and this hepatoma was studied.*

Groups of weaning Buffalo strain rats were pair-fed or ad libitum *fed a diet with and without B_6. The animals were inoculated with 7794A hepatoma cells at weaning and at different intervals afterwards and sacrificed* 49 *days after each inoculation. Tumor weight was over* 3 *times greater in* ad libitum *fed controls regardless of time of inoculation. Tumor weight in pair-fed controls inoculated after* 30 *days was also about* 3 *times greater than that of the vitamin B_6 deficient "partner" group.*

In general, the activity levels of TT and SD were lower in vitamin B_6 deficient animals. Four hours after administration of hydrocortisone significant rise in TT activity was noted in normal and host liver of vitamin B_6 deficient or non-deficient animals. Hormonal induction of TT was observed only in the tumors of animals fed the vitamin B_6 plus diet ad libitum. *Induction of TT activity in the tumors of pair-fed controls or tumors of animals fed the deficient diet was not observed.*

In the liver of the normal vitamin B_6 deficient rat, the rise in SD activity following induction by hydrocortisone was half that observed for the non-deficient animals. Hormonal induction of SD was not observed in hepatoma 7794A or host liver whether or not the animals received the vitamin B_6 deficient or supplemented diet.

INTRODUCTION

IT IS WELL known that vitamin B_6 (i.e. pyridoxine, pyridoxal, pyridoxamine and their phosphorylated derivatives) is required for the normal growth of young rats. It is also generally accepted that pyridoxal phosphate is the coenzyme of enzymes involved in transamination and decarboxylation reactions and possibly of certain other enzymes. The absence of this growth factor from the diet affects the activity of several enzymes in animal tissues [1–6] as well as the induction of enzymatic activity by steroids [2, 5, 6). Regression of growth of lymphosarcoma in mice has also been observed in the absence of the vitamin [7, 1]. Whether lack

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of vitamin B_6 also affects the growth of well differentiated hepatomas and the development of enzyme activity has not been investigated. This study reports the effect(s) of vitamin B_6 deficiency on (*a*) the growth of hepatoma 7794A, (*b*) the activity of tyrosine transaminase and serine dehydratase (two vitamin B_6-requiring enzymes) of normal and host liver and this hepatoma, and (*c*) the induction of these enzymes by hydrocortisone.

MATERIAL AND METHODS

Animals

Buffalo male rats weighing approximately 40–50 g were inoculated intramuscularly in the hind leg muscles with hepatoma 7794A cells, generation 39 [4]. The animals were housed individually in a windowless, air-conditioned room with an automatic on–off light switch. The lights were off from 8:00 p.m. to 8:00 a.m. and on from 8:00 a.m. to 8:00 p.m.

Vitamin B_6 deficiency was induced by feeding *ad libitum* weaning rats a diet deficient in vitamin B_6 but otherwise optimal (Nutr. Biochem. Corp., Cleveland, Ohio) as described previously [6]. Control animals were fed the same diet but their water was supplemented with pyridoxine HCL (4 mg/l) and the pH was adjusted to 6·3. Water was offered *ad libitum*. Average water consumption was 25–30 cm^3 in a 24-hr period. Control animals were either fed *ad libitum* or pair-fed. In pair feeding tests, animals of the same weight and age were "partnered" into pairs. One animal of each pair was fed the deficient diet *ad libitum* while his "partner" was allowed the same amount of diet as that which the animal on the deficient diet consumed the previous day. Food consumption was measured daily.

Determination of enzyme activity

All animals were sacrificed between 8:30 and 11:30 a.m. Livers and tumors were quickly removed and washed with cold 0·14 M KCl. Excess connective tissue was removed from each tumor and the weight recorded. Homogenization of tissues was carried out with a Blessing glass homogenizer (L–pestle clearance, 0·006 in.) in 7 volumes of cold 0·14 M KCl, as previously described [6, 8, 9]. Homogenates were centrifuged for 30 min at 4°C at 105,000 × *g* in the Spinco Model L ultracentrifuge. The clear supernatant was used to measure enzymatic activity.

Tyrosine transaminase, (L–tyrosine: 2–oxoglutarate aminotransferase, E. D. 2.6.1.5) activity was measured as previously described using the method of Diamondstone [10]. In some cases, the method of Canellakis and Cohen [11] was also employed, as previously described [12]. The enzyme unit is defined as that amount catalyzing the formation of 1 μmole of P-hydroxybenzaldehyde in 10 min at 37°C [10, 6].

Serine dehydratase, (L–serine hydrolyase, deaminating, E.C. 4.2.1.13) activity was determined as described by Suda and Nakagawa [13]. The enzyme unit is defined as that amount catalyzing the formation of 1 μmole of pyruvate per min at 37°C [13].

Protein was measured by the method of Lowry *et al.* [14]. Hydrocortisone hemisuccinate (Mann) was dissolved in 0·9% NaCl and given intraperitoneally four hours before sacrifice. The dosage of the hormone was 6 mg/100 g of body weight.

RESULTS

In attempts to decipher whether vitamin B_6 deficiency affects the growth of hepatoma 7794A as well as the development of enzymatic activity in host liver and this hepatoma, groups of male weanling Buffalo rats were fed *ad libitum* a diet lacking vitamin B_6 or supplemented with pyridoxine HCl. Further, a group of animals which were pair-fed to the animals receiving the deficient diet was also employed as an additional control. All animals were placed in the respective regimens at weaning when their body weight range was between 40–50 g. The three groups of animals, in general, may be identified as follows:

1A. All animals received the vitamin B_6 deficient diet *ad libitum* until sacrificed.

1B. All animals received the same vitamin B_6 deficient diet but pyridoxine HCl was also added to their water as stated in Material and Methods. This group was *pair-fed* to group 1A until sacrificed.

1C. All animals received the vitamin B_6 deficient diet but pyrodoxine HCl was also added to their water as stated in Material and Methods. This group was fed *ad libitum* until sacrificed.

Several tests were performed using this experimental approach. Groups of weanling animals of the type 1A, 1B, and 1C, as shown above, were inoculated with hepatoma 7794A cells and placed in their respective regimens at the same time, referred herewith as time zero

(t_0). Time zero always denotes the start of the experiment. In all tests reported here tumor growth and enzymatic activity were measured at the time of sacrifice or 47–49 days after inoculation.

The effects of the degree of vitamin B_6 deficiency on tumor growth was tested as follows. Groups of weanling animals of the types shown above were inoculated with tumor cells not only at time zero (t_0) but also after maintainance on their respective diets for different intervals, i.e. after 7, 15, or 31 days.

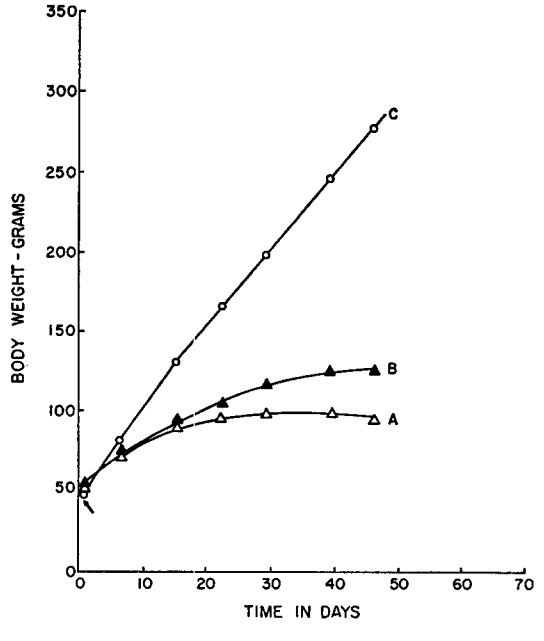


Fig. 1. *Average weight gain of male Buffalo rats fed a vitamin B_6 deficient diet* ad libitum *or a control (vitamin B_6 plus) diet on a restricted (pair-fed) or* ad libitum *basis. Groups of* 4 *or* 5 *weaning animals (groups A, B and C; Table* 1) *were inoculated with tumor cells at time zero (arrow) and were fed the vitamin B_6 deficient or control (B_6 plus) diet for the indicated time (MATERIAL AND METHODS). Group A: Animals received the deficient diet* ad libitum; *Group B: Animals received the vitamin B_6 plus diet but were pair-fed to group A. Group C: Animals received the vitamin B_6 plus diet* ad libitum.

The results of experiments are presented in Tables 1 and 2, and the accompanying Fig. 1. The average growth of hepatoma #7794A in terms of wet weight following the inoculation of tumor cells in the various animal groups is shown in Table 1. The average body weight of each group of animals at the time of inoculation and also at sacrifice, average liver weight and food consumed by each animal as well as the time of inoculation of the tumor cells are also shown in this Table. The table shows only representative data. Tumor growth was minimal in the groups of animals fed the deficient diet *ad libitum*. The pair-fed groups developed tumors with higher average weight than those of animals fed the deficient diet. The difference, however, was not statistically significant. Tumor weight was always highest in the group fed the control diet (plus pyridoxine) *ad libitum*. The average weight of the tumors from these animals was 3–4 times that of animals fed the deficient diet *ad libitum*. Data of this type were obtained whether the three groups of animals (1A, 1B, 1C, this section) were inoculated at the beginning of the experiment, time zero (t_0), or whether they were inoculated after 7 days (t_7) or after 15 days (t_{15}). The average tumor weight of groups of animals inoculated with tumor cells at the latter times (t_7 and t_{15}) was practically the same as the average tumor weight of the respective groups of animals inoculated at time zero (t_0). Therefore, only the results of the t_0 inoculation tests are shown here. Although the effect of feeding the vitamin B_6 deficient diet up to 15 days (inoculation at t_{15}) did not influence the average tumor weight, feeding first for 31 days and then inoculating with tumor cells resulted in significantly different average tumor weights. The average tumor weights of the pair-fed and the *ad libitum* fed groups (groups E and F, Table 1) were similar and the difference in the two weights was not statistically significant. On the other hand, the tumor weights of these latter two groups were approximately 3–4 times greater than the average tumor weight of the group fed the vitamin B_6 deficient diet group (D).

In all tests reported here the group of animals fed the deficient diet grew the least followed by the pair-fed and the *ad libitum* fed groups which received the pyridoxine supplemented diet. The accompanying Fig. 1 shows the growth pattern of different groups of animals of the type 1A, 1B and 1C mentioned previously (this section) which were inoculated with tumor cells at time zero (t_0). The growth patterns of the three groups of animals inoculated with tumor cells after 31 days on their respective regimens (t_{31}) were of similar nature as those of respective groups inoculated at time zero (t_0) and shown as A, B and C in Fig. 1. They were, however, heavier than similar groups inoculated at time zero (t_0) since these groups of animals were older by 31 days (Table 1).

In attempts to relate the effect of the absence of vitamin B_6 with tumor growth and metabolic activity the specific activity of two pyridoxal phosphate requiring enzymes, i.e. serine dehydratase and tyrosine transaminase, was measured in normal, host liver and hepatoma

Table 1. *Growth of Morris hepatoma* #7794A *in the absence and presence of vitamin* B_6 *and under* ad libitum *and pair-feeding conditions*

Group	No. animals	Diet	Time of inoculation	Duration of Tumor growth	Average weights (g) Body weight	Liver weight	Tumor weight	Body weight/ Tumor weight	Ave. food consumed g/rat per 24 hr
A	5	Vit. $B_6(-)$ *Ad libitum*	$t_0(50)$*	47	94 ± 14·0	4·0 ± 0·2(5)†	0·73 ± 0·2(4)‡	129	8·1 ± 2
B	4	Vit. $B_6(+)$ Pair-fed to A	$t_0(50)$	47	123 ± 5·8	2·5 ± 0·5(4)	0·91 ± 0·1(10)	135	8·1 ± 2
C	4	Vit. $B_6(+)$ *Ad libitum*	$t_0(50)$	49	278 ± 5·0	8·0 ± 0·4(4)	2·94 ± 0·4(7)	95	17·0 ± 4
D§	4	Vit. $B_6(-)$ *Ad libitum*	$t_{31}(80)$	48	154 ± 7·0	4·7 ± 0·5(4)	0·78 ± 0·5(5)	198	8·4 ± 2
E	5	Vit. $B_6(+)$ Pair-fed to D	$t_{31}(106)$	48	185 ± 1·0	4·8 ± 0·4(5)	2·40 ± 0·5(8)	77	8·4 ± 2
F	3	Vit. $B_6(+)$ *Ad libitum*	$t_{31}(160)$	48	348 ± 23·0	8·4 ± 0·6(3)	2·80 ± 0·5(6)	124	19·1 ± 5

* Number in parenthesis is the average body weight of the group at the time of inoculation.; t_0, t_{31} indicate the number of days the animals were fed the diets shown prior to inoculation with tumor cells.

† Number in parenthesis is the number of livers averaged.

‡ Number in parenthesis is the number of tumors averaged. Numbers following the ± sign are the standard error of the mean in all cases.

§ After 31 days on the $B_6(-)$ diet, pyridoxine was given for only 3 days. Each animal consumed approximately 360 μg of pyridoxine during this period.

Table 2. Effect of vitamin B_6 deficiency on tyrosine transaminase and serine dehydratase activities of normal, host liver, and hepatoma #7794A and on their induction by hydrocortisone

Regimen	Normal liver	Host liver	Hepatoma	Normal liver	Host liver	Hepatoma
	Tyrosine transaminase activity (units/mg protein)					
	− Hydrocortisone			+ Hydrocortisone		
1. Vit. B_6(−); *Ad lib.*	129±41(3)*	148±21(12)*	77±10(24)*	419±18(4)*	292±33(7)*	92±19(14)*
2. Vit. B_6(+) Pair-fed		163±(11)	78±6(22)	340±34(4)	236±42(7)	74±9(14)
3. Vit. B_6(+); *Ad lib.*	176±31(4)	173±18(8)	95±7(10)	365±30(3)	428±35(4)	150±15(8)
	Serine dehydratase activity (units/mg protein)					
1. Vit. B_6(−); *Ad lib.*	0·49±0·1(3)	0·43±0·2(4)	0·0(8)	1·85±0·3(4)	0·30±0·1(5)	0·01(10)
2. Vit. B_6(+); *Ad Lib.*	00·7±0·06(3)	1·10±0·2(2)	0·2(4)	2·9±0·3(2)	1·60±0·4(2)	0·0(4)

* Number in parenthesis is the number of animals per group, livers averaged or hepatomas averaged. All numbers following the plus or minus sign represent the standard deviation.

#7794A, in both vitamin B_6 deficient and non-deficient rats. The effect of hydrocortisone administration on the activity of these two enzymes was also examined. The results of these tests are shown in Table 2. In general, animals fed the vitamin B_6 deficient diet showed lower transaminase and dehydratase levels in all tissues examined. The administration of hydrocortisone to normal or tumor-bearing animals resulted in increased transaminase activity levels in normal and host livers of all groups of animals irregardless of regimen. The rise in transaminase activity above the uninduced level was not significant in hepatomas of animals fed the deficient diet or the control but restricted diet (pair-fed group). Hormonal induction of this enzyme occurred, however, in hepatomas of animals fed the vitamin B_6 control diet (plus pyridoxine) *ad libitum*.

Serine dehydratase activity was not observed in this hepatoma, generation 39, [4] by the assay method employed here [13]. In some tumors, however small but insignificant values were observed. Hydrocortisone failed to induce this enzyme in the livers of host animals fed the vitamin deficient diet. High serine dehydratase values were found in animals fed the control diet (plus pyridoxine) *ad libitum*. Induction of the enzyme in liver by hydrocortisone was also observed in these normal animals. The rise in SD activity above that of the uninduced level was 2× that observed in the deficient normal liver. SD activity was slightly induced in host liver and no induction of this enzyme was observed in this hepatoma. Other tests (to be reported alsewhere) showed that the rise in transaminase and/or dehydratase activities after the administration of the steroid was not observed in the presence of cycloheximide [19].

DISCUSSION

The results presented here demonstrate that the absence of vitamin B_6 from the diet affects the growth of hepatoma #7794A as well as the development and hormonal induction of enzymatic activity. Tumor growth was minimal in animals fed the deficient diet and maximal in animals fed the vitamin B_6 control (plus) diet *ad libitum*. Although hepatomas grown in animals receiving the latter regimen were on the average 4× heavier than those of deficient animals, the average weight of hepatomas developed by pair-fed controls depended on the time of inoculation of tumor cells. If inoculation was performed at the start of the experiment (time zero, t_0) there was no significant difference in average tumor weight between the deficient and pair-fed (control) animals. That the absence, however, of vitamin B_6 from the diet does indeed affect the growth of hepatoma #7794A is illustrated by

the following observation. Groups of animals received the deficient diet *ad libitum* for 31 days prior to inoculating them with tumor cells. Average tumor weights of 0·78, 2·40 and 2·80 g were developed by the deficient, pair-fed and *ad libitum* fed control groups, respectively. This finding shows that control animals on a restricted food intake (pair-fed group) require a longer time prior to inoculation with tumor cells to develop hepatomas of similar average weight as animals receiving the control diet (vitamin B_6 plus) *ad libitum*. The requirement for a longer feeding period may be necessary for the accumulation of vitamin B_6. The difference between pair-fed groups inoculated at time zero and after 31 days on the vitamin B_6 plus (control) diet was not the vitamin but only the time prior to inoculation. Since tumor cells grow at a faster rate than normal cells they apparently require more vitamin B_6.* Tumor cells, therefore, are in a manner forced to borrow the vitamin from normal cells at a "borrowing" rate governed by the rate of tumor cell growth. It is interesting to note that liver pyridoxal phosphate decreased steadily after ascites hepatoma cells were inoculated into animals and that the coenzyme content of the cancer cells increased as they proliferated [3]. In our hands hepatoma #7794A grows as good in pair-fed controls (receiving the restricted diet) as in *ad libitum* fed controls receiving the same diet but only if enough pre-feeding time is allowed the pair-fed animals prior to tumor cell inoculation. The requirement for the long pre-inoculation feeding period may be necessary for the animals to store a sufficient amount of vitamin B_6.

Our interest in learning about tumor metabolic function and its relation to the lack of vitamin B_6 led to examination of the activities of two pyridoxal phosphate-requiring enzymes, i.e. tyrosine transaminase and serine dehydratase, of both normal and vitamin B_6 deficient animals and of the effect of hydrocortisone on the induction of enzymatic activity (Table 2). The specific acitivity levels of the two enzymes in vitamin B_6 deficient animals were, in general lower than in the nondeficient animals. The absence of the vitamin from the diet, however, did not appear to affect the transaminase level significantly although it did affect the level of dehydratase. Further, induction of transaminase by hydrocortisone in this hepatoma was influenced by the lack of vitamin B_6. It is especially interesting that this enzyme was induced only in hepatomas of rats receiving the vitamin B_6 plus diet *ad libitum*. This latter observation further points to the importance of the coenzyme not only in the growth process of hepatoma #7794A but also in its metabolic activity. How the presence of the vitamin affects the induction process of this enzyme is not understood and further tests are planned in this regard. In a previous study [6] we reported significantly lower transaminase levels in vitamin B_6 deficient Wistar strain animals as compared to non-deficient animals. The difference between this study and the previous one [6] is the strain of rats used. The results suggest that the animal strain is directly involved in the expression of this enzyme and that the presence or absence of the coenzyme affects the transaminase level at least in the Wistar rat [6].

Potter and co-workers [15] have examined the activity of this enzyme in various Morris hepatoma lines. The reported activity values varied greatly from almost zero to even greater than that of normal liver. Also in many hepatoma lines transaminase activity did not respond to dietary protein intake. Host liver enzyme levels, on the other hand, were similar to that of normal liver and increased with dietary protein consumption. The response of the enzyme to hydrocortisone in different hepatoma lines also varied from complete unresponsiveness to significant increases [16]. The results presented indicate that the absence of vitamin B_6 from the diet does not particularly influence the induction of transaminase by hydrocortisone in normal or host liver of Buffalo strain rats but that it does influence the hormonal induction of the enzyme in hepatomas of animals fed the vitamin plus diet *ad libitum*.

Absence of vitamin B_6 from the diet had a more pronounced effect on serine dehydratase activity and its induction by hydrocortisone. In this case, minimal activity values were recorded for normal and host livers of vitamin deficient animals suggesting that the presence of the coenzyme may be necessary for the full expression of the activity of this enzyme. Furthermore, SD activity appears to be absent in this hepatoma, (generation 39 [4]), although in some instances negligible values were recorded. The hormonal induction of this enzyme was considerably influenced by the absence of vitamin B_6. The rise in activity in normal liver of deficient animals was about 50% less than

* Recent tests in our laboratory have shown that the pyridoxal phosphate content of hepatoma NO # 7777 was 5-fold greater than that of host liver in animals fed the deficient vitamin B_6 diet [20].

that observed for non-deficient animals. Further, in deficiency there was no induction of enzymatic activity in host liver and only a slight increase was recorded for host livers of animals receiving the vitamin B_6 plus diet. These observations indicate a requirement of this vitamin for the full induction of this enzyme in normal (no tumors) liver and further that the presence of the tumor interferes with the process of induction. As mentioned previously, it seems quite probable, that the tumor cells "borrow" the vitamin from the surrounding normal cells [3] and since the vitamin appears to be necessary for induction, its depletion by the tumor interferes with enzyme induction.

Potter *et al.* [15] reported a very low serine dehydratase activity level (10–15 μmoles per gram of tumor) in hepatoma 7794A, generation 18, which was not influenced by dietary protein intake. These workers used an automated assay method to determine enzymatic activity [17] which for technical difficulties we could not adopt in our laboratory. Instead, the enzyme assay method recommended by Suda and Nakagawa [13] was employed. In our hands, the presence of serine dehydratase activity in hepatoma #7794A, generation 39 [4] was not detected by this method. It is also of interest to note that Davis *et al.* [18] reported that in some hepatomas serine dehydratase levels were not measurable while in others were high and the higher the chromosome number of the hepatoma the higher the activity of this enzyme. The correlation of activity with chromosome number corresponds with the findings of Potter *et al.* [15]. Further, Watanabe *et al.* [16] reported an increase of this enzyme in hepatoma 7800 following injections of hydrocortisone for seven days. This, however, was not the case with other hepatoma lines examined by these workers.

Although the results presented here do not offer evidence for a detailed mechanism of vitamin B_6 action on tumor growth and metabolic (enzyme) function and its hormonal regulation (induction), nevertheless, the data demonstrate the necessity of this growth factor for the growth of hepatoma #7794A, the normal development of enzymatic activity and its hormonal induction.

REFERENCES

1. L. S. Dietrich and D. M. Shapiro. Antagonist studies: comparison of deoxypyridoxine and pyridoxine-deficiency therapy on various vitamin B_6 enzymes. *Proc. Soc. exp. Biol. (N.Y.)*, **84,** 555, (1953).
2. E. C. C. Lin, M. Civen and W. E. Knox. Effect of vitamin B_6 deficiency on the basal and adopted levels of rat liver tyrosine and tyrptophan transaminase. *J. biol. Chem.* **233,** 1183, (1958).
3. K. Ito, I. Nakahara and Y. Sakamoto. Vitamin B_6 metabolism of cancer cells and tumor bearing rat liver. *Gann,* **55,** 373, (1964).
4. M. Brin and V. F. Thiel. Relations between vitamin B_6 vitamin content and the activities of two trans-aminase enzyme in rat tissues at varying intake levels of vitamin B_6. *J. Nutr.* **93,** 213, (1967).
5. R. J. Milholland, R. Rosen, and C. A. Nichol. Inhibitory effects of 4-deoxypyridoxine on the endogenous activity and induction of tyrosine-α-ketoglutarate transaminase by cortisol. *Ann. N.Y. Acad. Sci. U.S.* **166,** 126, (1969).
6. G. P. Tryfiates. Hormonal and cofactor induction of tyrosine transaminase in vitamin B_6 deficiency. *Life Sci.* **10,** 1147, (1971).
7. R. C. Stoerk. The regression of lymphosarcoma implants in pyridoxine-deficient mice. *J. biol. Chem.* **171,** 437, (1947).
8. G. P. Tryfiates. Effect of benzene on rat liver poly-ribosomes. *Biochem. Pharmac.* **20,** 1669, (1971).
9. G. P. Tryfiates, R. F. Krause and J. K. Shuler. Transfer ribonucleic acid of vitamin A deficient rats. *Am. J. clin. Nutr.* **26,** 41, (1973).
10. T. I. Diamondstone. Assay of tyrosine transaminase activity by conversion of *p*-hydrophenylpyruvate to *p*-hydroxybenzaldehyde. *Anal. Biochem.* **16,** 395, (1966).
11. Z. N. Canellakis and P. P. Cohen, Purification studies of tyrosine-α-Ketoglutaric acid transaminase. *J. Biol. Chem.* **222,** 53, (1956).
12. G. P. Tryfiates and G. Litwack. Appearance of an increment of tyrosine aminotransferase activity in a cell-free system. *Biochem.* **3,** 1483, (1964).

13. M. SUDA and H. NAKAGAWA. L-Serine dehydratase (rat liver). *Methods in Enzymol.* **17B,** 346, (1971).
14. O. H. LOWRY, N. J. ROXEBROUGH, A. L. FARR, and R. J. RANDALL. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265, (1951).
15. V. R. POTTER, M. WATANABE, H. C. PITOT, and H. P. MORRIS. Systematic oscillations in metabolic activity in rat liver and hepatomas. Survey of normal diploid and other hepatoma lines. *Cancer Res.* **29,** 55, (1969).
16. M. WATANABE, V. R. POTTER, H. C. PITOT and H. P. MORRIS. Oscillations in metabolic activity in rat liver and hepatomas. Effect of hydrocortisone, glucagon, and adrenalectomy on diploid and other hepatoma lines. *Cancer Res.* **29,** 2085, (1969).
17. H. C. PITOT and N. PRIES. The Automated assay of complete enzyme reaction rates. I. Methods and Results. *Anal. Biochem.* **9,** 454, (1964).
18. J. L. DAVIS, H. J. FALLON and H. P. MORRIS. Two enzymes of serine metabolism in rat liver and hepatomas. *Cancer Res.* **30,** 2917, (1970).
19, G. P. TRYFIATES, J. K. SHULER and H. P. MORRIS. Enzyme activities in vitamin B_6 deficient, normal and tumor-bearing animals: Effect of hydrocortisone. *Proc. Soc. Exp. Biol. Med. (N.Y.), In press.*
20. G. P. TRYFIATES, F. L. SAUS and H. P. MORRIS. Hormonal induction of tyrosine aminotransferase activity in host liver and hepatoma NO #7777 in normal and cofactor depleted animals. (*Submitted for publication*).

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Cell Kinetics in Burkitt Lymphoma

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Abstract—*The cell kinetics of small pieces of diagnostic biopsies of African cases of Burkitt tumour was studied in* 13 *cases of primary tumour,* 4 *cases of recurring tumour and three cases of non-Burkitt lymphosarcomas in short time tissue culture with tritiated thymidine,* (3 *HTdr*) *in the medium. The* in vivo *uptake of* 3*HTdr was also studied in two patients in Stage IV. The uptake of* 3 *HTdr by tumour cells in ascites fluid was studied in one patient. In* 7 *patients Colcemid was given* 2–4 *hr prior to a biopsy, and the number of Colcemid-arrested mitoses counted. In* 4 *cutaneous nodules of Burkitt tumour, the actual growth rate was measured. The results were analysed by means of a mathematical tumour growth model (see Appendix by R. Bjerknes). The kinetic parameters observed were as follows: actual clinical doubling time* 66 *hr, labelling index* 17·34%, *rate of cells entering DNA-synthesis* 2·71% *per hr, mitotic index* 1·27%, *rate of cells entering mitosis* 1·75% *per hr, duration of* G_1 16·5 *hr, duration of DNA-synthesis* 6·5 *hr, duration of* G_2 2 *hr, duration of mitosis* 0·7 *hr, potential doubling time based on labelling indices* 25·6 *hr, potential doubling time based on number of Colcemid arrested mitosis* 39·6 *hr, cell loss in the* G_2 *phase* 30%, *total cell loss factor* 69% *of maximum possible cell gain.*

INTRODUCTION

BURKITT lymphoma is of considerable interest because of its rapid growth rate and its remarkable response to cytotoxic drugs [1]. Patients commonly present with large tumours which may have developed over a period as short as two weeks. Histologically the tumour is characterized by tightly packed areas of primitive lymphoid cells with cytoplasmic lipid containing vacuoles and scattered large histiocytes with signs of pronounced phagocytic activity, giving the tumour the so called "starry sky" appearance. The abundant macrophages with ingested cellular debris, and the frequent occurrence of areas of necrosis indicate considerable tumour cell death [2]. The presence of many mitotic figures may indicate a rapid growth rate. *In vitro* experiments using tritiated thymidine (3HTdr) have shown that Burkitt's lymphoma cells *in vitro* have a generation time of 19–36 hr [3,4,5]. In a preliminary report [14] we reported a potential doubling time of this tumour of 25·5 hr, based on 3HTdr labelling indices in fresh biopsy specimens. It has been suggested that the rapid rate of proliferation of Burkitt's lymphoma cells, combined with a high growth fraction of the tumour cells is responsible for the remarkable sensitivity to cytotoxic agents, and that it might be theoretically possible to achieve a total "tumour cell kill" with repeated administrations of drugs [10].

Our present state of knowledge of the proliferation kinetics of Burkitt lymphoma, however, has left many unanswered questions. The present study is an attempt to answer some of these.

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PATIENTS, MATERIAL AND METHODS

All patients with a histological and cytological diagnosis of Burkitt lymphoma admitted to the Lymphoma Treatment Center in Kampala, Uganda, East Africa between Sept. 1969 and May 1970 were eligible for study. In

addition, some patients seen in the autumn of 1970 were studied. The patients were clinically staged, treated according to a specific protocol, and followed closely thereafter [10]. Some few cases of non-Burkitt lymphoblastic lymphomas were also studied for comparison.

Short-time tissue culture with tritiated thymidine (3HTdr)

Prior to initial treatment (or in the case of relapse, to secondary treatment) fresh tumour tissue was removed at biopsy, and immediately immersed into tissue culture medium at 37°C, and cut into small blocks of about 1 mm^3. These were then placed in TC-199 containing 10% calf serum pH 7·35, and with 1μ Ci tritiated thymidine (specific activity 6·7 Ci/mM, New England Nuclear Corporation) per ml. To prevent cell division of labelled cells during a 4-hr period, 0·01 mg Colcemid (CIBA) was also added per ml medium. Ordinary small test tubes were used with 10 ml of medium in each tube. The tumour tissue blocks (4–10 in each tube) were incubated in a slowly revolving drum at 37°C, in atmospheric air, and specimens were removed at 1, 2, 3 and 4 hr. These were fixed, paraffin-embedded, and cut at 5μ. After coating with film emulsion, the number of labelled cells was counted in each specimen after 2 weeks exposure time. The specimens became somewhat edematous during tissue culture (Fig. 1) and labelling was found only in the superficial part of the block [15]. The counting of labelled cells thus had to be limited to the periphery of the sections, where areas of even labelling was found. The borderline between labelled and non-labelled areas was easy to determine (see also Discussion).

In vivo *uptake of 3HTdr*

Two patients in stage IV were given 3HTdr by intravenous injection every 6 hr during two days, amounting to about 0·5μ Ci per ml body volume per injection. Biopsies were taken at different time intervals. Specimens were treated as mentioned above, and the number of labelled cells counted. With this technique labelling was found throughout the tissue specimen and not only in the periphery.

Uptake of 3HTdr by cells in ascites fluid

Uptake of 3HTdr by Burkitt tumour cells in ascites fluid in one stage IV patient was studied. About 50 ml of ascitic fluid were withdrawn, and immediately spun down at 5000 rev/min for 10 min. These cells were resuspended in tissue culture medium with 3HTdr as mentioned above and incubated for two hours. Cells were removed at 1 hr, washed twice in saline, and spun down. Cytological smears were made, coated with film and the number of labelled cells counted.

Stathmokinetic method, mitotic counts

In 7 patients 10 mg Colcemid (CIBA) was given by intravenous injections 2–4 hr prior to a biopsy. The number of Colcemid arrested mitoses were counted among 1000 cells, and the mitotic rate determined. The mitotic count without Colcemid was calculated from 6 biopsies from patients who had Burkitt tumour but who had not been given Colcemid.

Measurement of actual growth rate

We studied two patients with cutaneous nodules of Burkitt tumour, using the method described by Steel *et al.* [8]. Altogether 4 cutaneous nodules were available for measurements in these two patients. We measured the greatest and the smallest diameters of these tumours with a caliper during 4 days. The products of these termed "tumour area" were calculated, and the tumour weights estimated. By the method of least squares growth rates and doubling times were calculated.

Mathematical calculations

These have been performed by research engineer Rolf Bjerknes, and are published as an Appendix to this paper.

RESULTS

Table 1 shows some clinical features, all 3HTdr labelling indices from the tissue culture studies, and the calculated potential doubling times in the tumours studied. In the upper part of the table are shown primary Burkitt tumours, arranged in order of decreasing proliferation rates. Then follow 4 cases of relapsing Burkitt tumours, and finally 3 cases of non-Burkitt lymphomas. The labelling indices after 1, 2, 3 and 4 hr respectively, are shown. These values are used for calculation of the regression line for each tumour. Finally, the potential doubling times (the time a tumour would have required to double its mass if all cells that synthesize DNA proceeded to mitosis and divided into two cells, and assuming no cell loss) are given in the last column. It is seen that the potential doubling time based on the number of cells incorporating 3HTdr, varies from 19·2 to 70·0 hr in different patients, with a mean of 25·6 hr. There is no difference between primary Burkitt tumours and relapsing tumours. Figure 2 shows a graph illustrating

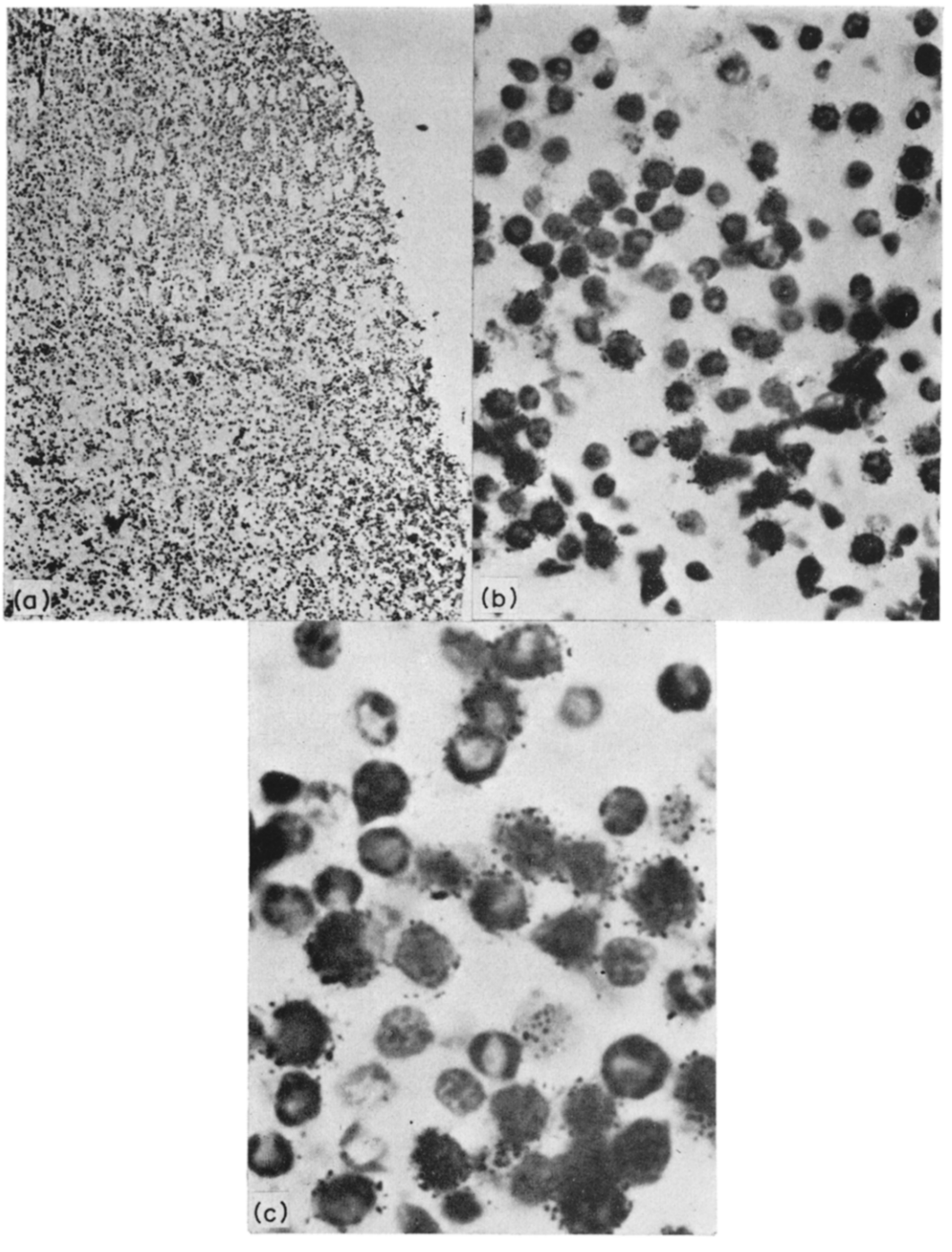


Fig. 1. (a) *Microphotograph of a piece of Burkitt tumour after* 4 *hr in tissue culture. Note the oedema and the loss of the histiocytes. Initial magnification* × 16.

(b) *Labelling in the peripheral zone of a piece of Burkitt tumour after* 4 *hr in tissue culture with* 3*HTdr. The periphery is at the bottom of the picture. The labelling decreases from bottom to top. Counting had to be performed in a narrow rim of the specimen, corresponding to about* 2/3 *of this picture. Initial magnification* × 160.

(c) *Heavy labelling in an area of the specimen. Initial magnification* × 320.

(to face p. 156)

Table 1. *Some clinical data and the labelling indices of fresh biopsies from patients with Burkitt tumour, and patients with non-Burkitt undifferentiated lymphocytomas, after* 1, 2, 3, *and* 4 *hr* in vitro *respectively*

Patient No.	Age/Sex	Stage	Biopsy site	Remission duration*	Survival*	Diagnosis	Labelling indices (% ± S.E.) 1 hr	2 hr	3 hr	4 hr	Regression line	*r*	Potential doubling time (hr)
16	11 F	III	Lymph node	48	72	BL	23·86 ± 1·65	22·95 ± 1·81	29·20 ± 1·60	39·69 ± 4·64	$Y = 15{\cdot}49 + 5{\cdot}38X$	0·90	12·9
5	8 F	II	Jaw	89+	89+	BL	23·05 ± 1·36	20·45 ± 1·92	32·45 ± 7·50	N.D.	$Y = 15{\cdot}92 + 4{\cdot}70X$	0·74	14·7
11	12 M	IV	Jaw	86+	86+	BL	21·92 ± 1·69	29·82 ± 3·06	29·45 ± 1·64	35·08 ± 2·08	$Y = 19{\cdot}30 + 9{\cdot}30X$	0·93	17·8
23	5 F	III	Ovary	4	24	BL	25·23 ± 1·89	26·18 ± 1·50	35·87 ± 2·52	33·82 ± 3·14	$Y = 21{\cdot}41 + 3{\cdot}55X$	0·85	19·5
3†	4 F	III	Ovary	6	16	BL	20·80 ± 1·99	21·12 ± 1·71	25·81 ± 5·27	30·24 ± 9·27	$Y = 16{\cdot}24 + 3{\cdot}30X$	0·95	21·0
20	4 M	III	Jaw	28	60	BL	29·83 ± 3·15	N.D.	36·42 ± 5·52	N.D.	$Y = 26{\cdot}53 + 3{\cdot}30X$	1·00	21·0
22	5 F	III	Jaw	8	65+	BL	17·50 ± 0·82	18·87 ± 1·77	23·75 ± 2·29	25·25 ± 2·97	$Y = 14{\cdot}34 + 2{\cdot}81X$	0·97	24·7
21	8 M	II	Jaw	68+	68+	BL	25·36 ± 2·01	31·68 ± 1·62	35·10 ± 2·45	33·29 ± 2·72	$Y = 24{\cdot}56 \pm 2{\cdot}72X$	0·83	25·5
2	4 M	IV	Jaw	22	55	BL	21·24 ± 1·60	26·29 ± 2·60	20·06 ± 1·84	31·81 ± 2·16	$Y = 18{\cdot}48 + 2{\cdot}55X$	0·61	27·2
12	7 F	IV	Ovary	4	33	BL	12·27 ± 2·48	19·49 ± 2·92	17·46 ± 3·05	20·83 ± 1·63	$Y = 11{\cdot}61 + 2{\cdot}36X$	0·81	29·4
19	11 F	III	Ovary	72+	72+	BL	20·85 ± 1·88	N.D.	22·45 ± 1·06	27·89 ± 1·49	$Y = 18{\cdot}05 + 2{\cdot}13X$	0·88	32·5
17	9 F	II	Jaw	45	77+	BL	26·38 ± 1·74	29·51 ± 1·33	29·37 ± 1·51	31·16 ± 1·74	$Y = 22{\cdot}55 + 1{\cdot}42X$	0·92	48·8
9	14 F	III	Ovary	8	10	BL	15·07 ± 1·15	17·92 ± 1·10	15·10 ± 0·83	19·31 ± 1·15	$Y = 14{\cdot}37 + 0{\cdot}99X$	0·60	70·0
				Mean			21·84 ± 1·89	24·03 ± 1·50	27·11 ± 2·78	29·85 ± 2·83	$Y = 18{\cdot}93 + 2{\cdot}71X$	—	25·5
14†	4 F	III	Ovary	4	24	BL (R)	24·75 ± 1·84	27·66 ± 3·04	30·34 ± 1·40	34·68 ± 1·57	$Y = 21{\cdot}24 + 5{\cdot}38X$	0·95	19·0
1	9 F	III	Subcutaneous	35	131+	BL (R)	15·08 ± 1·37	16·51 ± 1·35	24·68 ± 1·68	24·48 ± 1·21	$Y = 11{\cdot}10 + 3{\cdot}64X$	0·92	19·0
7	9 M	III	Testicle	30	127+	BL (R)	13·76 ± 1·12	21·31 ± 3·82	20·60 ± 1·68	21·30 ± 2·34	$Y = 13{\cdot}76 + 2{\cdot}19X$	0·77	31·7
10	12 F	III	Ovary	20	129+	BL (R)	14·53 ± 1·00	10·86 ± 1·01	11·65 ± 1·23	20·10 ± 1·24	$Y = 9{\cdot}87 \pm 1{\cdot}77X$	0·54	39·2
				Mean			17·03 ± 1·33	19·09 ± 2·31	21·82 ± 1·50	25·16 ± 1·59	$Y = 13{\cdot}99 + 2{\cdot}71X$	—	25·5
4	7 M	III	Jaw	92+	92+	LSA	26·56 ± 1·72	33·40 ± 1·84	41·97 ± 4·67	41·31 ± 3·37	$Y = 22{\cdot}61 + 5{\cdot}28X$	0·93	13·1
13	10 F	III	Retroperitoneal	—	40	LSA	23·47 ± 5·26	32·96 ± 3·41	29·20 ± 6·68	41·36 ± 3·35	$Y = 19{\cdot}26 + 4{\cdot}99X$	0·86	13·9
15	3 M	I	Jaw	40	52+	LSA	15·94 ± 5·93	N.D.	19·54 ± 1·27	24·08 ± 3·51	$Y = 12{\cdot}96 + 2{\cdot}58X$	0·97	26·9
				Mean			21·99 ± 4·30	33·18 ± 2·63	30·24 ± 4·21	35·58 ± 3·41	$Y = 20{\cdot}79 + 3{\cdot}78X$	—	18·3

The labelling indices are given from each patient ± standard error of the mean. On the bottom lines are given the mean values. The calculus for the regression line through all the 4 observation points can be seen, together with the correlation coefficient. The last column gives the calculated potential doubling times based on the rate of cells entering DNA-synthesis. *Weeks from initial therapy. †Patient No. 14 is the relapse in the same patient as No. 3. Other abbreviations: N.D.=not done; BL=Burkitt lymphoma; LSA=lymphosarcoma (poorly differentiated); *r*=correlation coefficient.

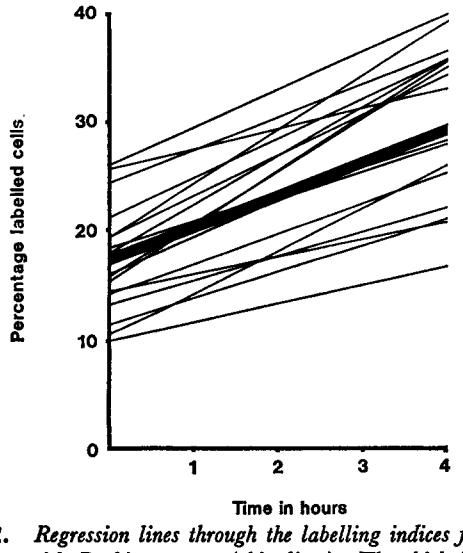


Fig. 2. Regression lines through the labelling indices for 17 patients with Burkitt tumour (thin lines). The thick line is the average regression line. The slope of this line is a measurement of the rate of cells entering DNA-synthesis in these tumours. For numerical values, see Table 1.

the results. The mean rate of cells entering DNA synthesis is 2·81% of all cells per hr. The rate of cells entering DNA synthesis in malignant lymphomas of non-Burkitt type was estimated to be 3·78% of all cells per hour, giving a potential doubling time of 18·3 hr.

Table 2 shows the results from 7 patients who have been given Colcemid before operation. The mitotic rates vary from 1·09 to 2·71 per cent of all cells per hr with a mean of 1·75% of all cells per hr. This gives a mean potential doubling time based on the mitotic rate of 39·6 hr. The discrepancy between the potential doubling times based on incorporation of 3HTdr and on the Colcemid method will be discussed below.

The mean mitotic count in Burkitt tumours from patients who have not received Colcemid was measured in 5 biopsies and found to be $1·27 \pm 0·20$.

The measurement of the growth of cutaneous nodules gave an average growth rate of 1·05% of all cells per hr and an actual doubling time of 66 hr, with wide variations. The measurements are shown in Table 3.

The Burkitt tumour cells from ascites in one patient showed a labelling index of 36·0% in tissue culture.

The results from the two patients in Stage IV with continuous labelling for two days is shown in Fig. 3. The form of this curve fits well with the assumption that the growth fraction in this tumour is 90–100% of all cells [6] (see also Discussion).

Based on the observations and on this assumption the following values can be given by means of a mathematical model (see Appendix by Rolf Bjerknes).

Actual clinical doubling time 66 hr (observed).
Actual clinical growth rate 1·05% of existing tumour mass per hour (observed).
Labelling index 17·34% of all cells (observed).
Rate of cells entering DNA synthesis 2·71% of all cells per hr (observed).
Mitotic index 1·27% of all cells (observed).
Rate of cells entering mitotis 1·75% per hour (observed).
Duration of G1 16·5 hr (calculated).
Duration of DNA synthesis phase 6·4–6·6 hr (calculated).
Duration of G2 2 hr (assumed).
Duration of mitosis 0·7 hr (calculated).
Mean time spent in S-phase + G2 phase 8·4 hr (calculated).
Mean time spent in G1 + mitosis 17·2 hr (calculated).
Potential doubling time based on labelling indices 25·6 hr (calculated).
Potential doubling time based on number of Colcemid arrested mitoses 39·6 hr (calculated).

Table 2. Some kinetic parameters from patients with Burkitt tumour injected with Colcemid before operation

Patient No.	Tumour localization	Time from Colcemid to biopsy	No. of vision fields	Mitotic count %	Mitotic rate (% per hr)	Potential doubling time (hr)
29	Abdomen	2 hr 0 min	58	5·431 ± 0·51	2·71	25·6 hr
24	Maxilla	2 hr 10 min	33	4·981 ± 0·58	2·29	30·3 hr
26	Abdomen	2 hr 30 min	52	5·361 ± 0·49	1·83	37·9 hr
27	Abdomen	4 hr 30 min	128	7·114 ± 0·53	1·58	43·9 hr
30	Abdomen	2 hr 0 min	58	2·972 ± 0·32	1·48	46·8 hr
25	Maxilla	3 hr 40 min	220	4·582 ± 0·43	1·28	54·1 hr
28	Abdomen	4 hr 0 min	52	4·378 ± 0·44	1·09	63·6 hr
Mean value				4·974 ± —	1·75	39·6 hr

The mitotic count and the mitotic rate is shown, together with the calculated potential doubling times based on the mitotic rates. On the bottom line are given the mean values.

Table 3. *Actual measurement of increase in size of* 4 *cutaneous nodules in patients with Burkitt lymphoma measured during* 4 *consecutive days*

Day	Parameter	Tumour I	II	III	IV
1	Dia mm	20 × 20	16 × 17	13 × 15	18 × 19
	Area mm²	400	272	195	342
	Weight g	3·5	2·0	1·2	2·8
2	Dia mm	22 × 23	20 × 21	15 × 16	19 × 19
	Area mm²	506	420	240	361
	Weight g	5·0	3·8	1·6	3·0
3	Dia mm	24 × 26	22 × 27	18 × 18	20 × 20
	Area mm²	624	594	324	400
	Weight g	6·9	6·4	2·6	3·5
4	Dia mm	25 × 27	23 × 29	20 × 22	21 × 22
	Area mm²	675	667	440	462
	Weight g	7·8	7·6	4·1	4·4
Mean of each tumour	Growth rate hr^{-1}	0·01052	0·01795	0·01620	0·00594
	Doubling time hr	65·91	38·61	42·78	116·71
Mean of all tumours	Growth rate hr^{-1}	0·0105			
	Doubling time hr	66			

For each day and each tumour is given the smallest and the largest diameter, the calculated tumour area and the calculated tumour weight. (For detailed explanation, see text.) In the two bottom lines are given the mean values from each tumour, and then the mean of all tumours.

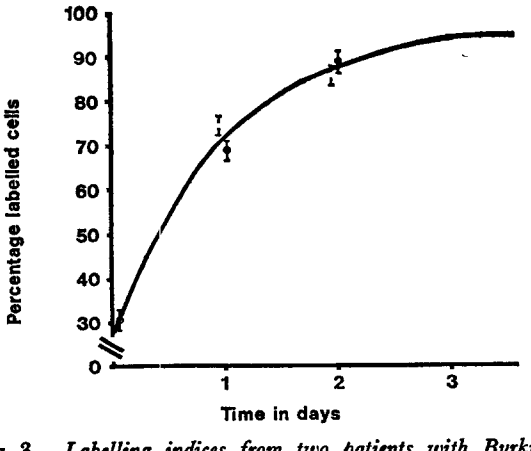


Fig. 3. *Labelling indices from two patients with Burkitt tumour in Stage IV, injected every* 6*th hour with* 3*HTdr during* 2 *days. Biopsy was taken* 1 *hr after the first injection, and then on the first and the second day. The bars represent* ± 2 *standard errors of the mean.*

Cell loss in the G2 phase 30% of all cells entering G2 (calculated, see Discussion).

Fraction of cells leaving G2 into mitosis 70% of cells entering G2.

Number of cells continuing from mitosis into G1 185·5% of the number of cells starting mitosis.

Cell gain after mitosis 85·5% of the maximal possible gain (14·5% loss factor in mitosis and G1).

Total cell loss factor 69% of the maximal possible cell gain calculated from all cells entering DNA synthesis.

When the kinetic parameters of each of these tumours were compared to the clinical data, no useful correlations were observed.

DISCUSSION

The measurement of the parameters of the cell population kinetics in a clinical tumour material is always difficult, partly due to the methods available, partly due to the inhomogeneity of the material, and partly due to the rules of medical ethics. In a disease as rare as Burkitt lymphoma, the number of cases available for study is always small. 3HTdr can be

used *in vivo* only in far advanced cases, and it is impossible to apply the percentage labelled mitosis technique since serial hourly biopsies from the same patient is totally unacceptable. In our material we therefore chose to perform tissue culture studies on small pieces of the diagnostic biopsy from each patient. We felt that it was acceptable to inject 3HTdr in two advanced cases in Stage IV, but Colcemid in 7 patients. The written consent of the parents was always obtained.

Measurements of uptake of 3HTdr of proliferating cells in organ culture present many difficulties. It is well known that DNA-synthesis goes on only in the periphery of the explants, independent of the time of incubation [15]. The depth of the active zone depends on the tissue, and can be increased if high oxygen pressures are applied. At Makerere University we had no access to high oxygen pressure in the tissue culture room, and we therefore had to count the number of labelled cells only in the periphery of the specimens, but it was easy to see where in each section labelling had occurred. The borderline between labelled and unlabelled area was rather well defined. Repeated countings of the same specimens were performed at month's intervals, and the results were always within very acceptable limits (deviations between two countings of the same specimen always below 5%). If the edema should have seriously interfered with the cell kinetics, one would have expected a lowering of the rate of 3HTdr uptake. This was not the case when compared to the Colcemid studies (see below). The narrow zone of oxygenated and therefore labelled cells severely reduced the number of viewfields available for study. Many sections from the same tissue block therefore had to be cut and studied. Another difficulty was that many of the biopsies turned out not be be from well preserved areas of the tumour. We often found areas of necrotic or partly necrotic tissue, making the counting of labelled cells per total number of cells difficult. The moderately small S.E. (Standard error of the mean), however, shows that our results might be accepted as useful guidelines, but certainly not as exact values.

With a stathmokinetic method mitoses can be blocked in the metaphase. By counting the relative number of arrested mitoses in a biopsy taken at a given time after the injection of the drug, the relative number of cells entering mitosis per time unit can be calculated, and used as an index for the rate of cell proliferation. We performed this in 7 patients, and found a rate of cellular proliferation based on the Colcemid method that was somewhat lower than that found with the 3HTdr method.

The discrepancies between the results obtained with 3HTdr and Colcemid may be explained in different ways:

(*a*) The 3HTdr method could give too high LI. It is realized that the tissue studied probably contains some cells that were at the end of the DNA-synthesis phase at the explantation time. It is generally accepted that new mitoses are never or rarely observed in short-term (4 hr) explants, but one cannot completely exclude the possibility that some cells could pass through G2 and mitosis, and divide before the tissue piece was fixed. This would give a too high value for the labelling index. We feel that this has been avoided by adding Colcemid to the tissue culture medium, thus preventing any cytokinesis during the 4 hr of tissue culture (see also below).

A very thorough comparison of *in vivo* and *in vitro* uptake of labelled thymidine has been performed by Helpap and Maurer [16]. These authors concluded that "The results for 8 different cell types (carcinomas, sarcomas, melanomas and normal liver tissue) show that the autoradiograms of excised tissue samples incubated with labelled thymidine represent the conditions of *in vivo* labelling. These experimental results obtained in animal experiments might be applied most probably to human biopsy samples too".

It is therefore reasonable to believe that the 3HTdr method is valid.

(*b*) The Colcemid-method may give too low values because the block is not complete or because of cell degeneration after arrest. The use of Colcemid to measure cell kinetics has been objected to by Tannock [11] who advocated a dose response and a linearity study being performed in each new experimental situation. Since such studies require serial biopsies and many test objects, it is obvious that this type of control cannot be performed in a study of human cases.

A clinical study of cell proliferation in human tumours from this institute utilizing Colcemid [12,13] was the first to demonstrate cell loss from human tumours as an important factor in tumour cell kinetics. The percentage of cell loss demonstrated by the Colcemid methods has been confirmed by many later studies.

The blocking effect of Colcemid is not of the same duration in all tissues. It is our experience that in rapidly dividing tissues

5 hr is too long for a straight line increase in the number of Colcemid-arrested mitosis. To avoid an error of this type, we took care to use shorter times between Colcemid injection and operation, as shown in Table 2. A 3 hr delay was preferred, but in the operation theatre this was not always possible. With this shorter time, a linear increase in Colcemid arrested mitosis is more probable. This short term also rules out the possibility of an appreciable amount of cell degeneration after arrest taking place. The mitotic count in tumours from patients not receiving Colemid was 1·27% of all cells. The mitotic duration is 0·7 hr, giving a mitotic rate *in vivo* of 1·81% of cells per hr. In the Colcemid study we found a mitotic rate of 1·75% of cells per hr. There is thus good correlation between the two results. Neither in the cultures with 3HTdr and Colcemid, nor in the patients given Colcemid, did we see any anaphases or telophases. Labelled mitoses were never seen. This is a strong indication that the Colcemid block was complete. It is therefore unlikely that an incomplete Colcemid block is the cause of the discrepancy between the proliferation rate calculated on the basis of labelled cells and on Colcemid arrested mitoses.

(*c*) We believe that the most probable explanation of the difference is that a certain cell loss takes place during G2. This means that some cells that have taken up 3HTdr during DNA-synthesis do not proceed to mitosis, but are delayed in G2 and die. Assuming this explanation, the cell loss in the G2 phase in Burkitt tumour cells can be calculated to about 30% of the cells entering DNA-synthesis.

The fact that the Colcemid method showed a lower rate of cellular proliferation than the 3HTdr method, may be important. Since the Colcemid method measures cell division, it is reasonable to believe that this method gives results nearer to the real rate of new cell production than the 3HTdr method, which has been used in most earlier studies.

The potential doubling time can be calculated from the rate of cellular proliferation. Based on the incorporation of 3HTdr, the potential doubling time of Burkitt tumour is on the average 25·6 hr, whereas the potential doubling time based on the Colcemid method is nearer to 40 hr. The discrepancy between these potential doubling times, and the actual doubling time of 66 hr, is probably due to cell loss which may largely take place in G2, but certainly also during mitosis and in G1.

There is much information about chromosomal abnormalities in Burkitt tumour cell lines, recently reviewed by Ikeuchi *et al.* [14] in connection with a cytogenetic tissue culture study. In 10·4–22·6% of the cells chromosomal abnormalities, such as breaks, fragmentations, chromatid exchanges, ring- or dicentric units and pulverizations were seen during metaphase. It is reasonable to assume that many of these abnormalities are lethal. From our study we calculated that about 15% of the cells die during mitosis and G1, a figure that fits well with the percentages of chromosomal abnormalities.

A complete knowledge of the cell kinetics of a tumour implies a determination of the size of the growth fraction. We have tried to estimate the growth fraction by continuous injection of 3HTdr in two patients in stage IV during two days. The result, as shown in Fig. 3, fits well with the assumption that the growth fraction in this tumour is 0·9–1·0. This judgement is based on a comparison of this curve with similar curves from experimental tumours where the growth fraction has been measured by the percentage labelled mitosis curve and continuous injection of 3HTdr [8]. A labelling index of 36 per cent after one hr incubation with 3HTdr of Burkitt ascites tumour cells points to a high rate of cell proliferation in the ascitic form of this tumour. It is difficult to understand this result if the fraction was not close to one.

The calculation of the clinical doubling time is based on the average growth of 4 cutaneous tumours in two patients during 4 days. It is obvious that by measuring only two diameters in such cutaneous nodules in only 4 tumours, the results are to be regarded with some reservations. Clinical experience, however, leads us to believe that a clinical doubling time of 66 hrs on the average is too rapid. The patients observed were also in Stage IV. Many Burkitt tumours grow more slowly than this. Thus it is probable that the real average cell loss is much higher than here calculated. Our results show the lowest percentages of cell loss hitherto seen in human rapidly growing tumours [9]. We are therefore of the opinion that the average clinical doubling time of Burkitt tumour is higher than 66 hr, and that the average cell loss rate is higher than 70% of the rate of cells entering the DNA-synthesis.

The number of mitotic figures observed in tumours without injection of Colcemid is based on representative biopsies from many patients

and is probably a reliable figure. From this and the Colcemid arrested mitoses it can be calculated that the duration of mitosis in these cells is 0·7 hr, a value that fits well with the results of others. A cell cycle time of about 26 hr of which G1 takes 16·5 hr, S 6·6 hr, G2 2 hr and M 0·7 hr also fits well with reported results (19–24 hr) from the study of cell suspensions of Burkitt tumour in tissue culture [3,4,5].

As regards the cell loss in this tumour, many histiocytes are seen, often filled with nuclear and cytoplasmic debris. This points to a high phagocytic activity by the histiocytes in these tumours. Necrotic areas and single necrotic cells are often seen. Electron-microscopic studies have not been performed by us, but the possibility of apoptosis also taking place [7] is certainly realistic.

Based on these considerations and on the assumption that the rate of DNA-synthesis in an explant immediately after biopsy mirrors the rate of DNA-synthesis *in vivo* [16], it may be concluded that the cell proliferation in Burkitt tumour is very rapid, but that there are great differences in the rate of cell renewal between different cases of Burkitt tumour. These variations were not dependent upon (i) the age of the patient, (ii) whether the tumour was primary or a recurrence, or (iii) the location of the tumour.

There seems to be general agreement that a good response to cytostatic treatment is most common in tumours with a high rate of cell turn-over. Kinetically this may explain why a single dose of a cytostatic drug reduces the tumour considerably; the cytostatic drug stops all cell proliferation, whereas the process of cell loss goes on at constant or increased speed. In two days then, most of the cells will have passed through their life cycle and die.

A reduction of tumour size, however, is not by itself a cure. Thus cultivation of biopsies *in vitro* from three patients with malignant lymphomas of non-Burkitt type gave an average potential doubling time of only 18·3 hr. There is thus not much difference between the Burkitt tumour and other lymphomas of non-Burkitt type with regard to this parameter. A reservation must of course be made due to the small number of cases analyzed, and the wide variation in the results. But since the rate of cellular proliferation seems to be similar, or even slightly faster in other undifferentiated lymphomas, our results cannot explain why Burkitt tumour seems to be more amenable to chemotherapy than other lymphomas. The size of the growth fraction was not estimated in the non-Burkitt lymphomas. A considerably lower growth fraction may possibly account for poorer responsiveness to treatment. Immunological mechanisms may certainly be responsible for the differences in the effect of the treatment.

REFERENCES

1. D. P. Burkitt and D. H. Wright, *Burkitt's Lymphoma.* E & S Livingstone, London, 1970.
2. S. Berard, G. T. O'Conor, L. B. Thomas and H. Torloni. Histopathological definition of Burkitt's tumour. *Bull WHO* **40,** 601 (1969).
3. E. H. Cooper, G. L. Frank and D. H. Wright, Cell proliferation in Burkitt tumours. *Euro. J. Cancer* **2,** 377 (1966).
4. E. H. Cooper, D. T. Hughes and N. E. Topping, Kinetics and chromosome analyses of tissue culture lines derived from Burkitt lymphomata. *Brit. J. Cancer* **20,** 102 (1966).
5. M. A. Epstein, Y. M. Barr and B. G. Achong, Preliminary observations on new lymphoblast strains (EB_4, EB_5) from Burkitt Tumour in a British and a Ugandan patient. *Brit. J. Cancer* **20,** 475 (1966).
6. I. F. Tannock, A comparison of the relative efficiencies of various metaphase arrest agents. *Exp. Cell Res.* **47,** 345 (1967).
7. J. F. R. Kerr, A. H. Wyllie and A. R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Brit. J. Cancer* **26,** 239 (1972).
8. G. G. Steel, K. Adams and J. C. Barrett, Analysis of the cell population kinetics of transplanted tumours of widley-differing growth rate. *Brit. J. Cancer* **20,** 784 (1966)
9. F. Gavosto and A. Pileri, Cell cycle of cancer cells in man. In *The Cell Cycle and Cancer* (Edited by R. Baserga) p. 99. Marcel Dekker, New York (1971).
10. J. L. Ziegler, R. H. Morrow, L. Fass, S. K. Kyalwasi and P. P. Carbone. Treatment of Burkitt's lymphoma with cyclophosphamide.*Cancer (Philad.)* **26,** 474 (1970).

11. T. IKEUCHI, J. MINOWADA and A. A. SANDBERG, Chromosomal variability in ten cloned sublines of a newly established Burkitt's lymphoma cell line. *Cancer (Philad.)* **28,** 499 (1971).
12. S. B. REFSUM, P. BERDAL and A. EVENSEN, Cell proliferation in human malignant and normal tissue. *Abstracts of the 9th International Cancer Congress, Tokyo,* p. 88 (1966).
13. S. B. REFSUM and P. BERDAL, Cell loss in malignant tumours in man. *Europ. J. Cancer* **3,** 335 (1967).
14. U. IVERSEN, O. H. IVERSEN, A. Z. BLUMING, T. L. ZIEGLER and S. K. KYALWASI Cell kinetics of African cases of Burkitt lymphoma. A preliminary report. *Europ. J. Cancer* **8,** 305 (1972).
15. M. F. RAJEWSKY, Zellproliferation in normalen und malignen Geweben: ^{3}H-Thymidin-Einbau *in vitro* unter Standardbedingungen. *Biophysik* **3,** 65 (1966).
16. B. HELPAP and W. MAURER, Autoradiografische Untersuchung zur Frage der Vergleichbarkeit des Einbaus von markierten Thymidin unter *in vivo*-Bedingungen und bei Inkubation von Gewebeproben. *Virch. Arch. Abt. B. Zellpath* **4,** 102 (1969).

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Exponential Growth

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Abstract—*In order to analyze numerical results from work with Burkitt tumours, a mathematical tumour growth model is proposed. It is shown that a formula derived directly from exponential growth is inadequate in cases with large cell loss. More precise formulae are derived together with approximations for practical use. Further it is shown that the formula* $LI = R_S . T_S$ *is approximately valid also in an exponentially growing population. The numerical results from the previous paper are analyzed and a combined loss factor of* 69% *of the total expected increase in cell number is found.*

INTRODUCTION

THE AIM of the present paper is to use the results from the biological experiments to calculate the numerical values of different relevant cell parameters. However, before this can be done it is necessary to list all the parameters and properties of the tumour growth and the relations between them, i.e. to make a model of the tumour growth. (For a more general discussion of such theoretical models, see [1] and [2]).

A Burkitt tumour may be assumed to be an exponentially expanding cell population in which a constant percentage of the proliferative cells enters the DNA-synthesis phase per time unit. There may be both cell differentiation and cell loss, but the population is assumed to be in an "exponentially steady state" with asynchronous divisions. This means that the growth fraction (No. of cells in cycle/No. of all cells) is constant and independent of time. The different lymphoblasts are further assumed to be histologically indistinguishable (except for actual mitoses), and thus all numerical values are referred to the total number of cells. The histiocytes are excluded from consideration. The growth fraction in Burkitt tumour is assumed to be 100%. The results of the present theoretical treatment are applicable to cell populations also containing differentiating cells with limitations as stated above.

The following calculations are based on mean values without any assumptions about the statistical distributions of the different parameters, and this is of course a limitation. Mean values based on human material are not exact, hence limiting more elaborate statistical examination.

Possible diurnal variations in the parameter values are not considered since this would be beyond the scope of this treatment. For tumours growing as rapidly as Burkitt tumour, it may be assumed that diurnal variations are of minor importance.

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MODEL

In the present case the model will be extremely simplified, but still of some practical use. We assume as a reasonable approximation that the tumour as a whole grows exponentially. Further we assume that all cells proliferate, i.e. the growth fraction is 100%. This means that there is only one type of cell present and there are no resting (G0) cells. The cells divide quite asynchronously, but have equal cycle parameters. There is some cell loss, of which a part seems to take place in the G2-phase. In accordance with this we assume that a proportion of the cells die after they have finished the DNA-synthesis, another proportion of the cells die at mitosis, and some cells may die in G1.

LIST OF SYMBOLS

The following symbols will be used:

T_d = actual clinical doubling time

G = growth rate $= \frac{\ln 2}{T_d}$ (1)

R = rate of proliferation

R_S = rate of cells entering the DNA-synthesis phase or S-phase

R_M = rate of cells entering the mitotic phase or M-phase

T_{G1} = meant time spent in the G1-phase

T_S = mean time spent in the S-phase

T_{G2} = mean time spent in the G2-phase

T_M = meant time spent in the M-phase

T_p = potential doubling time $= \frac{\ln 2}{R_M}$ (2)

T_v = virtual doubling time $= \frac{\ln 2}{R_S}$ (3)

T_2 = $T_S + T_{G2}$

T_3 = $T_M + T_{G1} = T_v - T_2$

N_S = $N_S(t)$ = Number of cells in the S-phase

N_M = $N_M(t)$ = Number of cells in the M-phase

N_G = $N_G(t) = N$ = number of generative cells = all cells

N_0 = size of the cell population at the time $t=0$

LI = labelling index $= \frac{N_S}{N}$

MI = mitotic index $= \frac{N_M}{N}$

α = fraction of cells dying in the G2-phase = number of cells dying relative to the number of cells entering.

β = fraction of cells dying in the M-phase = number of cells dying relative to the number of cells entering.

From these last two we may get the following derived variables:

$\gamma = 2\alpha$ = loss factor in the G2-phase

$(1-\alpha) = (1-\gamma/2)$ = fraction of cells entering from G2 into mitosis.

$\delta = 2\beta$ = loss factor in the M-phase

$2(1-\beta) = (2-\delta)$ = fraction of cells continuing into the G1-phase.

$(1-\delta)$ = cell gain after mitosis

$\lambda = \gamma + \delta - \frac{\gamma.\delta}{2}$ = total loss factor

$(1-\lambda)$ = total cell gain

For a stationary cell population the total loss is 100% of cell renewal, and for an increasing population the loss is between zero and 100%. (For a decreasing population the loss is more than 100%).

EXPONENTIAL GROWTH

The assumption about a constant growth rate may be stated as follows:

$$\frac{dN}{dt} = G.N \tag{4}$$

which gives:

$$N = N_0.\exp(G.t) \tag{5}$$

By setting: $N = N_1$ at a time $t = t_1$ and
$N = 2.N_1$ at a time $t = t_1 + T_d$

we get the formula:

$$G.T_d = \ln 2 \tag{6}$$

It is tempting to use the Equations (4)–(6) also in cases with cell loss in the following form:

$$\frac{dN}{dt} = (1-\lambda).R.N \tag{7}$$

which gives:

$$N = N_0.\exp[(1-\lambda).R.t] \tag{8}$$

And in analogy with Equation 6 we get the formula:

$$R.T_d = \frac{\ln 2}{(1-\lambda)} \tag{9}$$

For zero loss, i.e. for $\lambda = 0$, we get $R = G$, and the formula is obviously usable for very low values of the loss factor. However, we will try to show that the Equations (7–9) are not valid in cases with large cell loss, and further that Equation (9) with the same loss factor gives an approximation for R or T_d which is too high by a factor of $1/\ln 2$ or about 40%. It is obvious that the assumption about a homogenous population, which is the basis for Equation (9), is too simple in cases with large cell loss. In the present numerical example about one third of the population is engaged in the dividing process (the phases S, G2 and M), and this is a significant part of the population.

GROWTH WITH LOSS

In any asynchronous population, growing as a whole, the total number of cells will increase during any time interval, and also when some particular cells enter the S-phase and pass G2 into the M-phase. This is because the cells already present in the S- and G2-phase have to proceed to mitoses and divide and thereby increase the population. As the cells enter and advance through the S-, G2-, and M- phase they form a steadily decreasing proportion of the total number of cells, even without any cell loss occurring. Measurements of such a population ought to give R_M less than R_S provided the relative rate is constant. Any cell loss in the S- and G2-phase will accentuate the discrepancy.

When assuming that the tumour as a whole grows exponentially, then the following equations are valid:

$$R_S.(1-\gamma/2) = R_M.\exp(G.T_2)$$

$$R_M.(2-\delta) = R_S.\exp(G.T_3)$$

which gives:

$$\gamma = 2.\left[1 - \frac{R_M}{R_S}.\exp(G.T_2)\right] \tag{10}$$

$$\delta = \left[2 - \frac{R_S}{R_M}.\exp(G.T_3)\right] \tag{11}$$

The total loss is then:

$$(2-\lambda) = (1-\gamma/2).(2-\delta)$$

$$\lambda = \gamma + \delta - \frac{\gamma.\delta}{2} \tag{12}$$

By assuming that all loss takes place in the M-phase, we may set $\gamma = 0$ in Equation (10) and substitute the result into Equation (11). We then get:

$$\lambda = \left[2 - \exp(G.T_v)\right] = \left[2 - \exp\left(\frac{(\ln 2)^2}{R_S.T_d}\right)\right] \tag{13}$$

This result may be put into a form which gives straight lines in a log–log plot with λ as a parameter:

$$R_S.T_d = \frac{(\ln 2)^2}{\ln(2-\lambda)} \tag{14}$$

For zero loss, i.e. for $\lambda = 0$, we get $R_S = G$ just as for Equation (9). For a very small loss, i.e. for λ near to zero, Equation (9) is a good approximation to Equation

(14). It is also possible to show that the relative error introduced in this way will be approximately equal to:

$$\lambda \cdot \left(1 - \frac{1}{2.1n2}\right) \approx 0{\cdot}28.\lambda$$

In order to show this, the following approximations are usable:

$$\exp(x) \approx 1+x \quad \text{and} \quad 1n(1+x) \approx x$$

which are both valid for $|x| \ll 1$

For large loss, i.e. for λ near up to 1·0, it is possible in the same manner to show that the following approximations are usable:

$$\lambda = \left[1 - \frac{(1n2)^2}{R_S . T_d}\right] \tag{15}$$

$$R_S . T_d = \frac{(1n2)^2}{(1-\lambda)} \tag{16}$$

It may also be shown that the relative error introduced by using Equation (16) in place of Equation (14) will be approximately equal to

$$\frac{(1-\lambda)}{2}$$

Further it is seen when we compare the Equations (9) and 16 that they differ by a factor

$$1n2 = 0{\cdot}693 \approx 0{\cdot}7$$

which gives a substantial error when it is neglected.

In this way it has been shown that in cases with large cell loss the Equations (10–16) will give better results than Equation (9), although naturally not "correct" results.

PRACTICAL RESULTS

For cases with large loss the following formula will be usable for practical applications:

$$R . T_d = \frac{0{\cdot}02}{(1-\lambda)} \tag{17}$$

Here the rate R *is measured per hr*, and the doubling time T_d *is measured in days*. In such cases, when this approximate formula is used, there will be only a slight added error by using the mitotic rate, R_M, in place of R_S for the rate of proliferation, R. This procedure is used in such cases where only R_M is measured and it is known by other means that the loss in the G2-phase is insignificant.

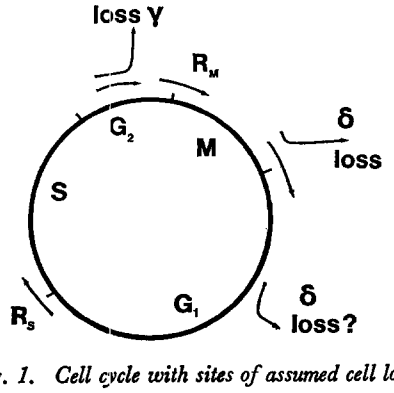


Fig. 1. Cell cycle with sites of assumed cell loss.

DURATION OF THE S-PHASE

For an exponentially and asynchronously growing population, an additional problem may be discussed. In calculations concerning stationary cell populations the following formulae have been used:

$$LI = R_S . T_S \quad \text{and} \quad MI = R_M . T_M$$

Are these formulae still valid for an exponentially growing population? The answer is yes, with a reasonable accuracy, based on the following line of reasoning: At the present moment with time $t = t_1$, the population consists of N cells and $N.R_S$ cells are entering the S-phase per time unit. The cells which have advanced a distance x into the S-phase did enter the S-phase at a time when the population consisted of $N.\exp(-G.x)$ cells, and $N.R_S.\exp(-G.x)$ cells did enter the S-phase per time unit. All cells in the S-phase are then:

$$N_S = \int_0^{Ts} N.R_S.\exp(-G.x).dx$$

$$LI = \frac{N_S}{N} = \frac{R_S.}{G}\left[1 - \exp(G.T_S)\right]$$

by setting:

$$q = \frac{LI.G}{R_S}$$

we get:

$$T_S = \frac{1}{G}.1n\left(\frac{1}{1-q}\right) \tag{18}$$

For small values of q, i.e. for $q \ll 1$, the following approximation is usable:

$$1n\left(\frac{1}{1-q}\right) \approx q$$

By using this we get the formula:

$$T_S = \frac{LI}{R_S} \text{ or } LI = R_S . T_S \tag{19}$$

and in the same manner:

$$T_M = \frac{MI}{R_M} \text{ or } MI = R_M . T_M \tag{20}$$

The approximation gives too small a value for T_S. The relative error is approximately equal to $q/2$. For instance for $q = 0{\cdot}1$ the error is about 5%.

NUMERICAL EXAMPLE

The following numerical values are given from basic assumptions or from the results of the study of the cell kinetic of Burkitt tumour:

Growth fraction = 100% (observation and assumption)

T_d = 66 hr (measured)

T_{G2} = 2 hr (assumption)

LI = 0·1734 (measured)

R_S = 0·0271 per hr (calculated)

MI = 0·0127 (measured)

R_M = 0·0175 per hr (calculated)

Then the following values may be calculated:

G = 0·0105 per hr from Eq. (1)

For the S-phase we have:

q = 0·0672 from Eq. (18)

$[T_S = 6{\cdot}6$ hr from Eq. (18)]

$T_S = 6{\cdot}4$ hr from Eq. (19)

The error introduced by the approximation is 3·4%.

For the M-phase we have:

$q = 0{\cdot}00762$ from Eq. (18)

$[T_M = 0{\cdot}7$ hr from Eq. (18)]

$T_M = 0{\cdot}7$ hr from Eq. (20)

The error introduced by the approximation is 0·38%.

$T_p = 39{\cdot}6$ hr from Eq. (2)

$T_v = 25{\cdot}6$ hr from Eq. (3)

$T_2 = 8{\cdot}4$ hr

$T_3 = 17{\cdot}2$ hr

$T_{G1} = 16{\cdot}5$ hr

$\gamma = 0{\cdot}589$ from Eq. (10)

$\gamma/2 = 0{\cdot}295 = 30\%$ of cells entering G2

$(1-\gamma/2) = 0{\cdot}705 = 70\%$ of cells entering G2

$\delta = 0{\cdot}145 = 15\%$ of the expected cell gain from Eq. (11)

$(2-\delta) = 1{\cdot}855$ of the expected 2·000 without loss

$(1-\delta) = 0{\cdot}855$

$\lambda = 0{\cdot}692 = 69\%$ of total expected cell gain from Eq. (12)

$[\lambda = 0{\cdot}692 = 69\%$ from Eq. (13)]

$[\lambda = 0{\cdot}731 = 73\%$ from Eq. (15)]

REFERENCES

1. A. B. Cairnie, L. F. Lamerton and G. G. Steel, Cell proliferation studies in the intestinal epithelium of the rat. II—Theoretical Aspects. *Exp. Cell Res.* **39,** 539 (1965).
2. G. G. Steel, K. Adams and J. C. Barrett, Analysis of the cell population kinetics of transplanted tumours of widely-differing growth rate. *Brit. J. Cancer,* **20,** 784 (1965).

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An Assessment of the Immunosuppressive Activity of the Anti-Tumour Compound 4′-[(9-acridinyl)amino]methanesulphon-m-anisidide (m-AMSA)*

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Abstract—*The experimental anti-tumour compound* 4′-[(9-*acridinyl*)-*amino*] *methanesulphon-m-anisidide* (*m-AMSA, NSC* 156303), *which is highly active against experimental tumours, suppressed the direct plaque-forming cell* (*PFC*) *response to sheep red blood cells in mice. The maximum tolerated dose had no effect on the PFC response when injected before or up to* 12 *hr after injection of antigen, yet it suppressed the response by* 99·5% *when injected* 28 *hr after the antigen. A more complete assessment of the suppression by this agent was gained by using spleen cell transfer and spleen cell culture systems. Mice pre-treated with cyclophosphamide* (120 *mg*/*kg*) *which behave as B-cell deficient animals, were used as recipients for cell transfer. Treatment of either the donor mouse or donor spleen cells with m-AMSA showed that B cells changed from a drug-insensitive to a drug-sensitive state between* 4 *and* 27 *hr after contact with antigen. Similar deductions could be made from experiments where the PFC response was measured in cultured spleen cells which were pre-treated with the drug m-AMSA may be useful to distinguish different developmental stages of the precursors of PFC.*

INTRODUCTION

IN RECENT years Cain *et al.* [1,2] have synthesized a number of heteroaromatic anti-tumour compounds which have shown marked activity against the L1210 murine leukaemia. One of these compounds is 4′[(9-acridinyl) amino] methanesulphon-m-anisidide, (m-AMSA, NSC 156303, structure shown in Fig. 1), which has been found to be at least as effective as cytosine arabinoside in treating L1210 leukaemia and has shown promise in a number of other animal tumour systems [2]. It has been accepted by the National Cancer Institute, U.S.A., as a candidate drug for clinical trials (H. B. Wood, Jr., N.C.I., personal communication) and its effects on a number of cellular systems are being investigated in this laboratory.

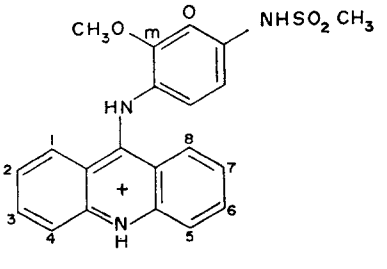


Fig. 1. *Structure of m-AMSA.*

Since the final elimination of tumour cells during or following chemotherapy may involve a host immune response [3] it is important to assess the effects of an anti-tumour drug on the immune system. We have investigated the effects of m-AMSA on the production of haemolytic plaque-forming cells (PFC) in mice in response to immunisation with sheep red blood cells (SRBC). The degree to which an

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anti-SRBC response is suppressed by some cytotoxic drugs depends on the method of assay [4]. We have used a combination of whole animal, adoptive cell transfer and cell culture techniques to assay the immunosuppressive capacity of m-AMSA (Fig. 2). These methods have allowed particular attention to be paid to the timing of drug treatment. The results obtained with m-AMSA reveal some unusual characteristics when compared with those reported for other drugs.

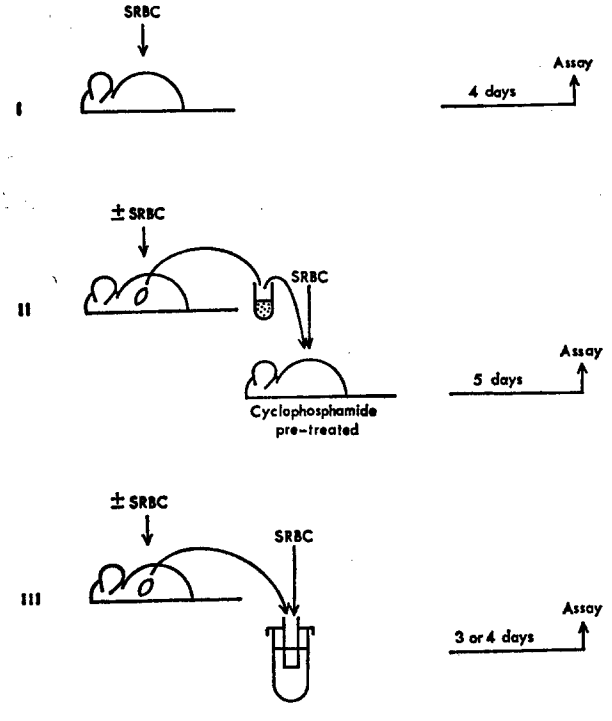


Fig. 2. Schematic representation of alternative techniques used for assessing immunosuppressive activity. Cytotoxic drugs can be injected into mouse at various times before and/or after SRBC as in I. Cytotoxic drugs can be injected into donor mouse at various times before or after SRBC in II or, alternatively, added to cells in an in vitro *incubation mixture before cell transfer to cyclophosphamide pre-treated recipients. Cultured spleen cells in III can be exposed to cytotoxic drugs either initially with subsequent washing and culturing or continuously for the duration of incubation.*

MATERIAL AND METHODS

Animals

F1 hybrid mice (C_3H/HeJ female $\times DBA_2$/J male) of age between 2 and 3 months and obtained from the Cancer Chemotherapy Laboratory, Cornwall Geriatric Hospital, Auckland were used for *in vivo* experiments. CBA mice were used for culture experiments and were maintained at Cornwall Geriatric Hospital and at the Department of Cell Biology, University of Auckland.

Cells

Sheep erythrocytes, suspended in Alsever's solution, were obtained from Auckland Hospital. The standard challenge, to induce a primary response in mice, was 3×10^8 erythrocytes injected intraperitoneally in 0·3 ml of phosphate buffered saline solution. Spleen cells from mice were prepared by teasing out spleens with forceps in balanced salt solution buffered with HEPES(N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) as used by Shortman [5]. Suspensions were drawn through a 20 gauge needle and allowed to settle for 10 min. Clumps of cells were discarded and cell suspensions were used for intravenous injection into the tail veins of recipient mice or in cell cultures.

Drugs

Cyclophosphamide (Endoxan Asta) was generously provided by Bristol Myers Co. Pty Ltd. Solutions were made up in saline immediately before use. m-AMSA and related agents (as the methane sulphonate salts) were provided by Dr. B. F. Cain, Cancer Chemotherapy Laboratory, Cornwall Geriatric Hospital, Auckland. They were dissolved and injected in water. All drugs were injected intraperitoneally in a volume of 0·2–0·3 ml.

Assay for antibody forming cells

Cells from mouse spleens were dispersed in buffered balanced salt solution, diluted as required in the same buffer, and assayed using the method of Cunningham and Szenberg [6] with minor modifications. Results were expressed on a logarithmic scale. Variability was indicated by using the standard error of the logarithms of the numbers of plaque forming cells (PFC).

Cultures

The immune response of mouse spleen cells in culture was generated as described previously [7]. When the effect of m-AMSA on *in vitro* immune responses was assessed, the drug was included in the medium of both the reservoir and the culture chamber.

RESULTS

Effects of m-AMSA and related compounds on the primary immune response in animals

m-AMSA is strongly anti-leukaemic when given either as a daily dose (5 mg/kg per day) or as a single dose (20 mg/kg) [2]. When administered to mice at a dose of 5 mg/kg on

days 0, 1, 2 and 3 after SRBC, m-AMSA inhibited the primary PFC response by 98%. A single dose of m-AMSA (20 mg/kg 24 hr after SRBC) inhibited the response by 99% (Table 1). The single dose of m-AMSA was found to inhibit the response and not merely delay it, since assay of PFC on days 6 and 7 still showed an inhibition of 97%. It was of interest to examine the effect of some drugs which were chemically related to m-AMSA [8 and unpublished data] eight of which were active and two inactive against the L1210 leukaemia. A correspondence between anti-leukaemic activity and immunosuppressive activity was observed (Table 1) and subsequent experiments utilised only m-AMSA.

Table 1. *Immunosuppressive activity of compounds related to m-AMSA*

	Dosage	Immuno-suppression¶	L1210 activity**
m-AMSA	4*	89%	+
o-AMSA‡	16*	0%	–
AMSA§	16*	80%	+
2-methyl AMSA‖	16*	0%	–
3-methyl AMSA	16*	99·4%	+
4-methyl AMSA	16*	97%	+
m-AMSA	20†	99%	+
3-methyl m-AMSA‖	20†	97%	+
4-methyl m-AMSA	60†	99·8%	+
3,5-dimethyl m-AMSA	60†	99%	+
4,5-dimethyl m-AMSA	150†	99%	+

* Dosage by intraperitoneal injection on days 0, 1, 2 and 3 after injection of 3×10^8 SRBC.
† Dosage by intraperitoneal injection 28 hr after injection of SRBC.
‡ 4′-((9-acridinyl) amino)-methanesulphon-o-anisidide.
§ 4′-((9-acridinyl)amino)-methanesulphonanilide.
‖ Methyl substituents on the acridyl ring are numbered as in Fig. 1.
¶ PFC produced on day 4 after SRBC as a percentage of the control.
** Biological data from Cain and co-workers [2,8], and unpublished data.

The effect of varying the timing of administration of m-AMSA was studied (Fig. 3). PFC were assayed 4 days after injection of SRBC. When a single dose (20 mg/kg) of m-AMSA was given before or up to 12 hr following an injection of the antigen, no suppression was evident. On increasing the time between the injection of SRBC and m-AMSA beyond 12 hr, the PFC response was suppressed, the maximum effect being observed with a time interval of 28 hr. Administration of the drug 92 hr after immunisation (4 hr before assay) had no effect.

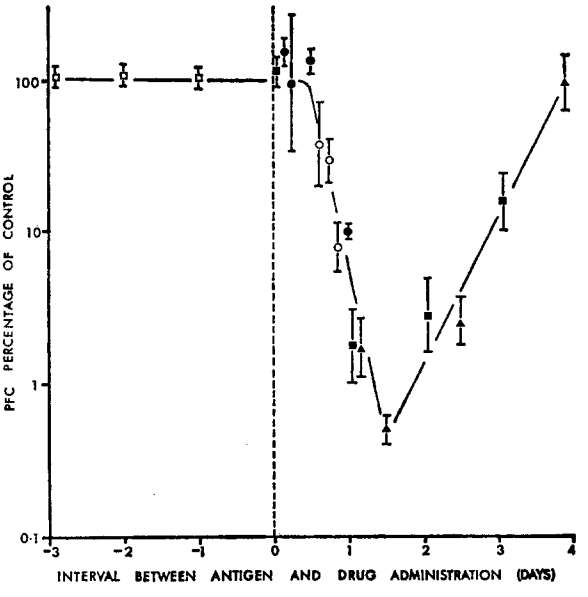


Fig. 3. *Effect of time of administration of the drug m-AMSA on the degree of immunosuppression. Mice were injected with* 3×10^8 *SRBC and PFC were assayed* 4 *days later. m-AMSA was injected intraperitoneally at a dose of* 20 *mg/kg at the time indicated. The number of PFC per spleen in different experiments is shown by different symbols. The standard error for each point is shown by a vertical line.*

Since 28 hr after antigenic challenge was the time for maximum immunosuppression by m-AMSA, the effect of different doses of drugs was examined when administered at that time. No effect was observed at doses of m-AMSA up to 5 mg/kg, but above this, the PFC response decreased exponentially with increasing drug dosage (Fig. 4). The drug also caused a reduction in the number of nucleated cells per spleen with a maximum effect on days 2–3. The number of cells in the spleen at the time of assay for PFC is included in Fig. 4.

Effect on m-AMSA on the PFC response in cell transfer experiments

If isolated spleen cells are injected together with SRBC into animals which have been treated with cyclophosphamide (120 mg/kg) 24 hr previously, a linear relationship between the number of cells inoculated and the PFC produced is obtained [4]. In the present experiments, the immune response in the recipient animals, measured 5 days following cell transfer, was about 2000 PFC/10^6 nucleated spleen cells injected. In the absence of added spleen cells, the recipient animals produced about 10^3 PFC per spleen.

If the donor animals were injected with SRBC (3×10^8 intraperitoneally) 48 hr before isolation of spleen cells and injection into cyclophosphamide pre-treated recipients, then about 500 PFC/10^6 spleen cells were

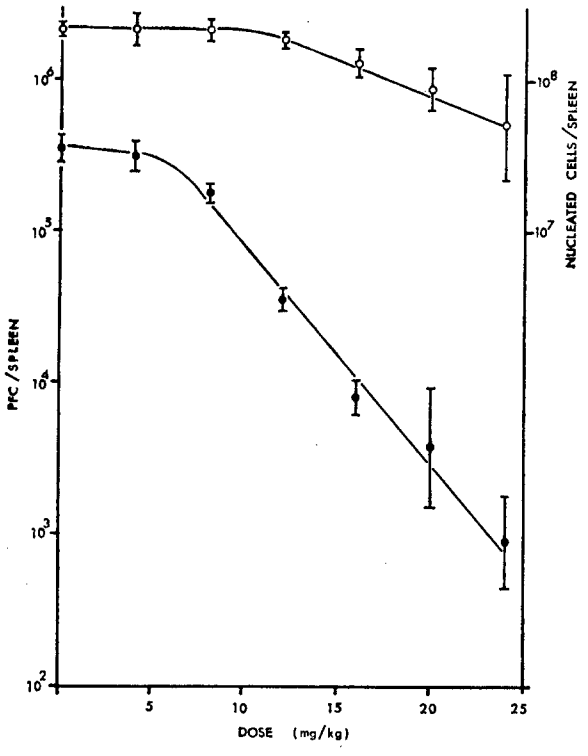


Fig. 4. Effect of dose of m-AMSA on the degree of immunosuppression. Mice were injected with 3×10^8 *SRBC and PFC were assayed 4 days later. Various doses of m-AMSA were injected intraperitoneally 28 hr after immunisation. The number of nucleated cells in the spleen was measured at the time of assay (open circles) and the number of PFC per spleen is shown by closed circles. The standard errors are shown by vertical lines.*

produced. In the present results, such donor animals are termed "primed" animals, whereas those which have received no prior injection of SRBC are termed "unprimed" animals. The effects of m-AMSA were determined by treating "primed" or "unprimed" animals with the drug at various times before transfer of the donor spleen cells to cyclophosphamide pre-treated recipients (Table 2).

(*a*) *Treatment of donor animals with m-AMSA.* Treatment of "unprimed" donor mice with m-AMSA (20 mg/kg) 2, 21 or 44 hr before cell transfer caused a slight increase in the number of PFC produced in the recipient animals per 10^6 cells transferred. However, a reduction in the number of nucleated cells per spleen was also caused by m-AMSA, if the number of PFC produced was divided by the fraction of the donor spleen transferred the number of PFCs produced per spleen—equivalent transferred could be calculated. A 40% reduction in this figure was evident 21 and 44 hr after injection of m-AMSA.

"Primed" donor mice were also treated with m-AMSA 2, 21, or 44 hr before cell transfer (i.e. 46, 27 or 4 hr, respectively, after injection

Table 2. Effect of pre-treatment of "primed" or "unprimed" donor mice with m-AMSA on ability of donor spleen to transfer immunity

Time after treatment with m-AMSA	PFC produced in cyclophosphamide-treated recipients: "Unprimed" donors PFC per 10^6 cells injected	"Primed" donors PFC per 10^6 cells injected
2 hr	1,960	55
21 hr	2,090	83
44 hr	2,950	500
Control	1,890	485

"Primed" donor animals were injected with 3×10^8 SRBC 48 hr before cell transfer. "Unprimed" donor animals received no SRBC. Donors were treated with m-AMSA at the indicated times before cell transfer. Recipient animals, injected with cyclophosphamide 24 hr beforehand, received an intravenous injection of 2×10^7 spleen cells followed by an intraperitoneal injection of 3×10^8 SRBC. PFC were measured 5 days later. Results are corrected for PFC produced by recipient animals which received no spleen cells.

of donors with SRBC). The number of PFC produced in recipient animals following cell transfer was markedly reduced when donors were treated with m-AMSA 2 or 21, but not 44 hr before cell transfer (Table 2). Exposure of spleen cells to m-AMSA 4 hr after immunisation with SRBC did not affect their ability to transfer immunity to cyclophosphamide pre-treated recipients 44 hr later, but exposure 27 or 46 hr after immunisation suppressed this ability.

(*b*) *Treatment of donor spleen cells* in vitro, *before cell transfer.* Since *in vivo* exposure of donor spleen cells to m-AMSA demonstrated a difference in sensitivity between cells from "primed" and "unprimed" animals, the exposure of spleen cells *in vitro* was investigated. The effects were compared to those obtained following incubation with high specific activity tritiated thymidine. This treatment is known to kill cells in the DNA synthetic phase of the cell cycle [9]. Spleen cell suspensions (8×10^7 cells/ml) in buffered balanced salt solution were prepared and warmed to 37°. m-AMSA was added from a stock aqueous solution (2 mg/ml) to a concentration of 10 μg/ml and the cells were incubated for 15 min at 37°. Other samples were incubated in the absence of m-AMSA, and in the presence of ^{3}H-thymidine (16 Ci/mM, Radiochemical Centre, Amersham) at a concentration of 50 μCi/ml.

Incubation of spleen cells from "primed" animals in the absence of m-AMSA had no effect on the ability of such cells to function

when transferred to mice pretreated with cyclophosphamide. 2×10^7 cells were transferred. Incubation with m-AMSA caused 96% reduction in the ability of such cells to produce PFC in recipient mice. Incubation with high specific activity ^{3}H-thymidine caused a 75% reduction. Incubation of spleen cells from "unprimed" animals with m-AMSA (10 μg/ml, 37°, 15 min) reduced their ability to produce PFC in recipient mice by 40%.

Effect of m-AMSA on the immune response of cultured spleen cells

The inhibitory effects of m-AMSA were investigated using cultures of spleen cells grown for 3 or 4 days under conditions which have been described previously [7]. When cultures were exposed continuously to m-AMSA, cultures of spleen cells from "primed" mice were less sensitive (at concentrations less than 1 μg/ml) than cultures of cells from "unprimed" mice (Fig. 5). Both types of cultures were inhibited strongly (95–99%) at concentrations of 1–10 μg/ml. In contrast, if spleen cells were exposed to m-AMSA in buffered balanced salt solution (10 min at 37°), then washed before culturing for 3–4 days, cultures of spleen cells from "primed" animals were inhibited to a greater extent than were cultures of spleen cells from "unprimed" animals (Fig. 6).

DISCUSSION

In view of the interactions between immune cells and tumour populations it is of considerable interest to examine the immuno-suppressive activity of drugs which have been selected for their antitumour properties. Because both T and B cells are involved in immunity [9] a complete assessment of immunosuppression by a drug must involve the examination of its effect on each cell type. The experiments carried out in this work concentrate on the cells of the "B" cell lineage.

The present results indicate that m-AMSA suppresses the PFC response in mice only when administered at certain times after immunisation. The peak PFC response, occurring 4 days after injection of SRBC, was not affected by injection of m-AMSA before or up to 12 hr after antigen. The most effective time of administration was 28 hr after antigenic challenge, when the PFC response was suppressed by 99·5%. Most other cytotoxic drugs, with the exception of some alkylating agents, act

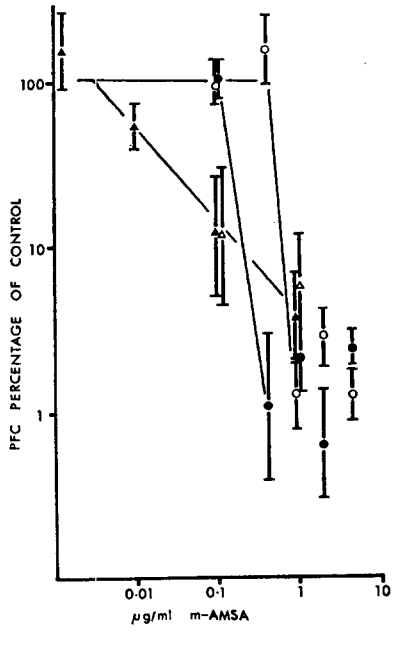


Fig. 5. The effects of addition of m-AMSA to cultures of spleen cells from "primed" (3×10^8 SRBC 48 hr previously) and "unprimed" mice. 1×10^7 spleen cells were cultured for 3 days in the presence of the indicated concentration of m-AMSA. The results are shown of two experiments using cells from "primed" animals (○, ●) and two experiments using cells from "unprimed" animals (△, ▲). The results are expressed as a percentage of controls, with standard errors shown by vertical lines. The number of PFC per control cultures was 3300 (○), 1700 (●), 180 (△), *and* 130 (▲).

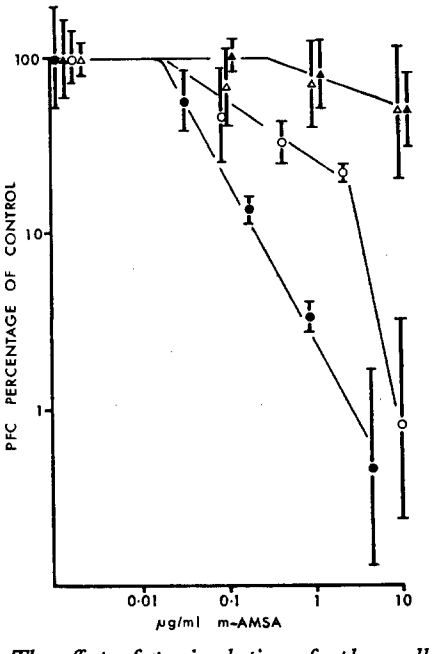


Fig. 6. The effect of pre-incubation of spleen cells from "primed" (3×10^8 SRBC 48 hr previously) and "unprimed" mice with m-AMSA on their ability to produce PFC in culture. Spleen cells were incubated with m-AMSA (10 μg/ml) *for* 10 *min at* 37° *in buffered balanced salt solution and subsequently washed and cultured at* 10^7 *cells/culture. Results are shown for cells from "primed" animals assayed at* 3 *days* (○, ●) *and cells from "unprimed" animals assayed at* 3 *days* (△) *and* 4 *days* (▲). *The results are expressed as a percentage of the control culture PFC which were* 875 (○), 1530 (●), 115 (△), *and* 650 (▲).

most effectively when administered 1–2 days after immunisation [10]. However, at doses used for chemotherapy of cancer in experimental animals, most of the drugs show some activity when administered before or at the same time as the antigen [10]. The data in Table 3 summarises some of the published data for suppression of the production of PFC.

Table 3. Effects of different immunosupressive agents administered at the same time as SRBC, on the PFC response after 4 days

Agent	Dose	Percentage inhibition of response	Reference
m-AMSA	20 mg/kg	0%	This work
actinomycin D	0·2 mg/kg	95%	12
cyclophosphamide	165 mg/kg	Tolerance	13
cytosine arabinoside	2500 mg/kg	98%	14
thioguanine	40 mg/kg	99·8%	15
vinblastine	16 mg/kg	99·98%	16

Since a number of structural relatives of m-AMSA were available, some of which were active and some inactive against the L1210 murine leukaemia [16] it was of interest to determine whether anti-leukaemic and immunosuppressive activity were linked. Results for 8 active and 2 inactive 4′-[(9-acridinyl)amino]-methanesulphonanilide derivatives showed a complete correspondence between anti-L1210 activity and ability to inhibit the PFC response (Table 1).

Cyclophosphamide-treated mice have previously been used as recipients in adoptive transfer experiments [17]. We have found that animals receiving a cyclophosphamide dose of 120 mg/kg are particularly useful recipients, since essentially only the "B" cell population is eliminated [18,19]. The dose response relationships between normal spleen cells and PFC produced indicates that no other cell types are limiting factors [19]. The adoptive cell transfer system measures the specific "B" population when normal spleen cells are transferred to mice treated with cyclophosphamide on the previous day.

Cyclophosphamide treated recipients were used to measure the effect of m-AMSA on the ability of donor spleen cells to transfer immunity. Donor animals were injected with the drug at different times before cell transfer (Table 2). Alternatively, isolated spleen cells were incubated with m-AMSA *in vitro*. The results suggest that B cells are insensitive to m-AMSA before or 4 hr after immunisation, but become sensitive by 27 hr after SRBC injection.

Other cell transfer systems which have been used to study the effects of cytotoxic drugs include transfer into irradiated mice or newborn rabbits. Sterzl [20] measured the effects of a number of agents in the latter system, and found that 6-mercaptopurine and 6-thioguanine treatment of recipients inhibited antibody formation following cell transfer from non-immunised rabbits. Effective inhibition by 6-mercaptopurine required that the drugs was injected at the same time as, or 24 hr after, cell transfer. Syeklocha *et al.* [16] using irradiated mice as recipients, showed that *in vitro* treatment of donor spleen cells with vinblastine was much more inhibitory to cells from mice which had previously been exposed to antigen. Kaufman [21] used irradiated recipient mice to examine the effects of actinomycin D on the ability of spleen cells to transfer PFC, and demonstrated an effect on B cells.

In vitro cell culture techniques were also used to investigate the action of m-AMSA. Isolated spleen cells were incubated with m-AMSA directly before culturing, and the drug concentration required for 50% inhibition of the PFC response was measured. This concentration was 0·04 and 0·15 μg/ml (2 experiments) for cells from "primed" animals, whereas it was 10 μg/ml for cells from "unprimed" animals (Fig. 6). This result is similar to that obtained in cell transfer experiments. However the results of experiments where m-AMSA was present continuously were unexpected (Fig. 5). At a concentration of 0·1 μg/ml, m-AMSA had little effect on PFC production by spleen cells from mice which had previously received SRBC, whereas it inhibited by almost 90% the PFC production by spleen cells from non-immunised animals. The reason for this behaviour is unknown.

A study of the selectivity of drug action must involve the size of the dose and the persistence of the drug within the biological system. The abrupt transition from m-AMSA-insensitive to -sensitive stages during the development of PFC is probably the result of two factors. Firstly, m-AMSA has a preferential effect on dividing cells, and secondly, it has a short biological half-life. Studies on the effects of m-AMSA on L1210 leukaemia cells have shown that cytotoxic activity persists in the animal for about 60 min but that all stages of the cell cycle are affected by a single exposure to the drug [22]. The *in vitro* incubation

experiments reported here suggest that all stages in the cell cycle of antigen-stimulated B cells are sensitive to m-AMSA. When isolated spleen cells were exposed to the drug for 10 or 15 min and then either cultured *in vitro* or transferred to cyclophosphamide recipients, a 96–99% inhibition of subsequent ability to produce PFC was obtained. In contrast, *in vitro* exposure of such cells to high specific activity ^{3}H-thymidine, a treatment known to be specifically lethal to "S-phase" cells [9], inhibited the ability of the cells to produce PFC by 75%.

It is possible that B cells, following antigenic stimulation, pass from a stage which does not permit to one which does permit the uptake of m-AMSA. The use of *in vitro* techniques for investigating antibody production has allowed the delineation of distinct steps within the differentiation of specific "B" cells [23]. The well-defined temporal change in sensitivity to this drug during a primary response suggests that m-AMSA may be of great value in extending investigations on "B" cell differentiation, using drugs sensitivity as a marker for discrete populations. The apparent lack of effect on "untriggered" cells and the almost complete suppression of a continuing immune response is an attribute of m-AMSA which may be of considerable value in designing effective dose schedules for this antitumour agent.

REFERENCES

1. B. F. Cain, G. J. Atwell and R. N. Seelye, Potential antitumour agents. X. Bisquaternary salts. *J. med. Chem.* **12,** 199 (1969).
2. B. F. Cain and G. J. Atwell, The experimental antitumour properties of three congeners of the acridylmethanesulphonanilide (AMSA) series. *Europ. J. Cancer.* (In press).
3. G. Mathé, Active Immunotherapy. *Adv. Cancer Res.* **14,** 1 (1971).
4. J. Marbrook and B. C. Baguley, The recovery of immune responsiveness after treatment with cyclophosphamide. *Int. Arch. Allergy* **41,** 802 (1971).
5. K. Shortman, N. Williams and P. Adams, The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions. *J. Immunol. Meth.* **1,** 273 (1972).
6. A. J. Cunningham and A. Szenberg, Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology* **14,** 599 (1968).
7. J. Marbrook, Primary immune response in cultures of spleen cells. *Lancet* **ii** 1279 (1967).
8. G. J. Atwell, B. F. Cain and R. N. Seelye, Potential antitumour agents. 12. 9-anilinoacridines. *J. med. Chem.* **15,** 611 (1972).
9. A. J. Becker, E. A. McCulloch, L. Siminovitch and J. E. Till, The effect of differing demands for blood cell production on DNA synthesis by hemopoietic colony-forming cells of mice. *Blood* **26,** 296 (1965)
10. *Transplant Rev.* **1,** 1–149 (1969).
11. T. Makinodon, G. W. Santos and R. P. Quinn, Immunosuppressive drugs. *Pharmacol. Rev.* **22,** 189 (1970).
12. P. Abramoff, C. Hintzke and N. Brien, Effect of actinomycin-D on antibody synthesis by spleen cells of immunized rats. *J. reticuloendothel. Soc.* **5,** 498 (1968).
13. A. C. Aisenberg, Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. *J. exp. Med.* **125,** 833 (1967).
14. J. E. Harris and E. M. Hersh, The effect of 1-β-D-arabinofuranosylcytosine on the immune response of mice to sheep red blood clells. *Cancer Res.* **28,** 2432 (1968).
15. M. C. Berenbaum, Immunosuppressive agents and the cellular kinetics of the immune. In *Immunity, Cancer and Chemotherapy.* (Edited by E. Mihich) pp. 217–237 Academic Press, New York, (1970).
16. D. Syeklocha, L. Siminovitch, J. E. Till and E. A. McCulloch, The proliferative state of antigen-sensitive precursors of hemolysin-forming cells, determined by the use of the inhibitor, vinblastine. *J. Immunol.* **96,** 472 (1966).
17. G. W. Santos and A. H. Owens, Adoptive transfer of immunologically competent cells. I. Quantitative studies of antibody formation by syngeneic spleen cells in cyclophosphamide pretreated mice. *Bull. Johns Hopk. Hosp.* **118,** 233 (1966).

18. L. W. POULTER and S. L. TURK, Proportional increase in the θ carrying lymphocytes in peripheral tissue following treatment with cyclophosphamide. *Nature* (*New Biol.*) **238,** 17 (1972).
19. J. MARBROOK and B. C. BAGULEY, Manuscript in preparation.
20. J. STERZL, The effect of immunosuppressive drugs at various stages of differentiation of immunologically competent cells. In *Immunity, Cancer and Chemotherapy*. (Edited by E. MIHICH) pp. 73–99 Academic Press, New York, (1970).
21. S. J. KAUFMAN, Selective inhibition of cells in the immune response by actinomycin D. *J. Immunol.* **106,** 781 (1971).
22. W. R. WILSON, B. F. CAIN and B. C. BAGULEY, (Manuscript in preparation).
23. J. S. HASKILL and J. MARBROOK, The *in vitro* immune response to sheep erythrocytes by fractionated spleen cells: biological and immunological differences. *J. immunol. Meth.* **1,** 43 (1971).

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Early Appearance of "Transformed" Cells from the Kidneys of Rats Treated with a "Single" Carcinogenic Dose of Dimethylnitrosamine (DMN) Detected by Culture *In Vitro*

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Abstract—*After three days on a sugar and water diet, inbred Wistar rats received a single carcinogenic dose of dimethylnitrosamine (DMN). Kidney cells were grown in monolayer cultures* in vitro *from both treated and control animals at various intervals from* 20 *hr to seven days, following treatment. Cells isolated from treated rats as early as* 20 *hr following treatment had a prolonged life-span* in vitro, *exhibited morphological transformation and an increased mitotic index, produced colonies in methylcellulose gels, showed a high relative plating and growth efficiency and were agglutinated in the presence of Concanavalin A.*

INTRODUCTION

THE NITROSO group of compounds contain some of the most potent carcinogens known to man [1–3] and are probably widely distributed in the environment [4–6]. In rats a single carcinogenic dose of dimethylnitrosamine (DMN—60 mg/kg body weight) has been shown to initiate the formation of kidney tumours in all individuals which survived the initial toxic insult [7–9]. Since one can apparently guarantee that every treated animal will develop a tumour, this experimental system should provide a suitable means of studying cells in the early stages of transition from the normal to the neoplastic state. Because all the DMN is eliminated from the body within 24 hr [10] one might expect that a single carcinogenic dose of the nitrosamine would result in cells being committed to a malignant course within this time. In this study we have attempted to establish the earliest stage at which one can detect growth and behavioural changes in cells from a target organ following exposure to a single carcinogenic dose of DMN. An *in vitro* approach to this problem was not possible because some earlier studies which involved direct treatment of cells *in vitro* with dialkylnitrosamines failed to produce toxic changes or transformation in the cells [11–13]. The apparent failure of the dialkynitrosamines to produce transformation following treatment of cells *in vitro* may reflect the relative stability of these compounds at neutral pH [10,14] and the possibility that they may require enzymically catalysed decomposition in the body [15,16] prior to exerting their carcinogenic effect. To circumvent this difficulty, in this study we have combined the *in vivo* DMN-treatment of the host animal with the *in vitro* isolation of the target organ cells [17].

The selection of a rat kidney cell system for a combined *in vivo/in vitro* study of chemical carcinogenesis has two distinct advantages over

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other systems. First, it has been established that a single dose of DMN can give rise to kidney tumour formation in every rat surviving the initial toxic effects of the nitrosamine [8]. Second, rat cells are less likely to transform spontaneously when grown *in vitro* than either mouse or hamster cells which are more commonly used for *in vitro* carcinogenesis studies [18,19].

MATERIALS AND METHODS

Wistar rats, five to six weeks old, were kept on a sugar and water diet for 72 hr then experimental animals received a single intraperitoneal injection of 60 mg DMN in 0·9% saline/kg body weight as previously described [8] and control animals received a single intraperitoneal injection of 0·9% saline. Animals were killed and the kidneys removed aseptically at 20 hr, 24 hr, 3 days, 5 days and 7 days following treatment with DMN. Fifteen DMN-treated and ten control rats were used in the following experiments.

Cell culture

After detachment of the kidney capsule, cortical tissue was removed and placed in warm Hank's Balanced Salt Solution (B.S.S.), cut into 2 mm fragments, washed thoroughly in Hank's B.S.S. and trypsinized in fluted flasks at room temperature in 10 ml aliquots of 0·125% trypsin in calcium and magnesium-free phosphate buffered saline. The flasks were gently agitated manually for 30 min and the supernatant discarded. This process was repeated five times and on the final two occasions, the supernatant was collected and transferred to conical centrifuge tubes containing 2 ml of growth medium (Weymouth's medium—Burroughs Wellcome & Co. Ltd., Rosebury, N.S.W. + 10% foetal calf serum–C.S.L., Parkville, Victoria) and centrifuged at 500 ***g*** for 5 min. The cells were resuspended in growth medium to give a concentration of 1×10^5 cells/ml and dispensed in 4 ml aliquots to 30 ml screw-capped tissue culture flasks (Falcon Plastics) and the cultures incubated at 37°C in an atmosphere of 5% CO_2 in air.

Six replicate cultures were established from each rat, the medium changed twice weekly and subcultures made when the cells had formed a complete monolayer, at intervals of 10–14 days following primary isolation, and 5–7 days thereafter. Cultures were examined daily using a Wild phase-contrast, inverted microscope.

Transformation frequency and mitotic counts

For mitotic and transformed-colony counts the medium was removed from confluent monolayer cultures and the cells washed twice in warm Hank's B.S.S. prior to adding 2–3 ml of Bouin's fixative and allowed to stand for 15 minutes at room temperature. The cells were then stained with haematoxylin and eosin and mounted in Epon (Shell).

Plating/growth efficiency

Cell monolayers were treated as for subcultivation and serial dilutions of the suspension of separated cells were made in growth medium to give cell counts of 100 cells/ml. A 0·2 ml aliquot of cell suspension was dispensed into each well of a 96 well microtest tissue culture plate (Falcon Plastics Cat. No. 3040). Plates were placed in a sealed air-tight plastic box in an atmosphere of 5% CO_2 in air and incubated at 37°C for seven days. The medium was then removed from each well and the well washed with three changes of Hank's B.S.S. prior to fixing the cells in 70% methyl alcohol, staining with Giemsa and counting the surviving healthy cells only, using a binocular (Wild) dissecting microscope. Plating and growth efficiency was expressed as the percentage of cells inoculated that were present after seven days in culture.

Colony formation in "Methocel"

A 2 ml layer of 0·9% Difco Bacto Agar in growth medium was placed into 30 mm dia tissue culture Petri dishes (Falcon plastics) and allowed to solidify at room temperature. Cells for investigation were removed from their cell culture flasks using a trypsin/versene mixture and after centrifugation were filtered through sterile gauze and resuspended in a soft gel of 1·16% carboxymethylcellulose [20] (Methocel MC Standard 4000 counts/ses—The Dow Chemical Co.—Michigan, U.S.A.) in growth medium to give a separated single-cell suspension of 5×10^3 cells/ml. Two ml of this cell suspension were placed in each of the agar-containing dishes which were then incubated for seven days at 37°C in an atmosphere of 5% CO_2 in air. Colonies were counted using a Wild dissecting microscope and an indirect light source. The colonies were seen as grey-white circumscribed masses in the orange-red "Methocel" gel.

Cytoagglutination

Cells which had been growing *in vitro* for more than seven weeks were removed from the culture flasks using 0·002% versene in P.B.S. Cells were then washed three times in P.B.S.

and resuspended to give a concentration of 4×10^6 cells/ml in P.B.S. Concanavalin A (Calbiochem.) was prepared in concentrations of 10 μg/ml and 100 μg/ml in P.B.S. An aliquot of 0·25 ml of cell suspension and 0·25 ml of Concanavalin A (Con. A) solution were gently mixed in a conical centrifuge tube and allowed to stand at room temperature for five minutes. A drop of the mixture was placed on a clean glass slide and examined using a Zeiss phase-contrast microscope. Controls were established using cell suspensions in P.B.S. only. Agglutination was scored according to the size of the cell aggregates.

RESULTS

The *in vitro* behaviour and properties of kidney cortical cells isolated from DMN-treated rats were compared with those of control untreated animals and the following observations made.

Cell survival, transformation and mitotic index

Figure 1 portrays cell survival in the various cultures and shows that the control cells ceased to grow at the third or fourth subculture and died out three to six weeks after isolation. With one exception, the cells from the DMN-treated rats however, have grown continuously in culture since isolation. In experiment B5 (Fig. 1) the actively growing cells from the DMN-treated animal were collected and stored in liquid nitrogen. In experiment B14 the cells from DMN-treated rats ceased to grow and died out at the same time as the corresponding control cells. The reason for this single failure to establish a continuously growing culture has not been determined but did not appear to be due to contamination. At intervals between five and seven weeks following isolation, transformation was observed only in the cells from DMN-treated rats. Transformed colonies of cells (Fig. 2) appeared with a transformation frequency of between 4·6 and 8·8 transformed colonies per 10^4 cells seeded (Table 1.) Following morphological transformation there was a marked increase in the number of observed mitoses in cells from DMN-treated rats compared with pre-transformation and control values (Table 2).

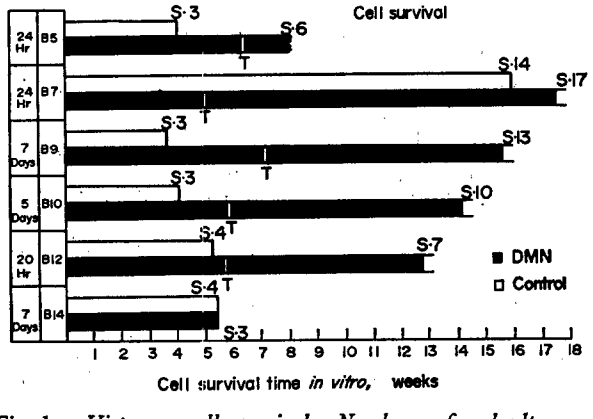


Fig. 1. *Histogram-cell survival. Numbers of subcultures carried out are indicated on the histogram prefixed by the letter S. The white lines (T) in the black areas indicate the stage at which transformation was first observed in the cultures. The time which had elapsed between treatment of the rat and primary isolation of the cells is indicated in the extreme left hand column with the experiment code number adjacent to it. The open ended histogram columns indicate that the relevant cultures were healthy and growing at the time of writing. The closed histogram columns indicate that the relevant cells have ceased to grow. In Experiment B5, the jagged end to the hatched column represents freeze storage of the cells while in the growing state.*

Table. 1 *Transformation frequency*

Rat kidney cells experiment number		Transformed colonies *W*2 mm dia/10^4 cells seeded
Controls	(B5,7,9,10, 12)	0
DMN	B5	6·0
DMN	B7	8·8
DMN	B9	7·2
DMN	B10	5·8
DMN	B12	4·6

Cultures from 5 control and 5 DMN-treated rats were fixed and stained and the transformed colonies greater than 2 mm dia were counted. Four replicate culture dishes of control and DMN cells were counted for each experiment.

Plating efficiency and growth ability

Cells from DMN-treated animals showed a steady and rapid increase in efficiency of plating from two weeks post-isolation onwards, whereas the control cells showed only a slight increase to three weeks and fell off rapidly to zero, six weeks after isolation (Fig. 3).

Colony formation in "Methocel" gel

Only cells from DMN-treated animals were able to form colonies in carboxymethylcellulose gels. From Fig. 4 it can be seen that there was a steady increase, with time, in the number of colonies formed following isolation. In addition, the cells isolated 24 hr after DMN-treatment had consistently lower colony counts than the cells isolated seven days after DMN-treatment.

Cytoagglutination with concanavalin A

Cells from four DMN-treated rats and two controls were tested for agglutination in the

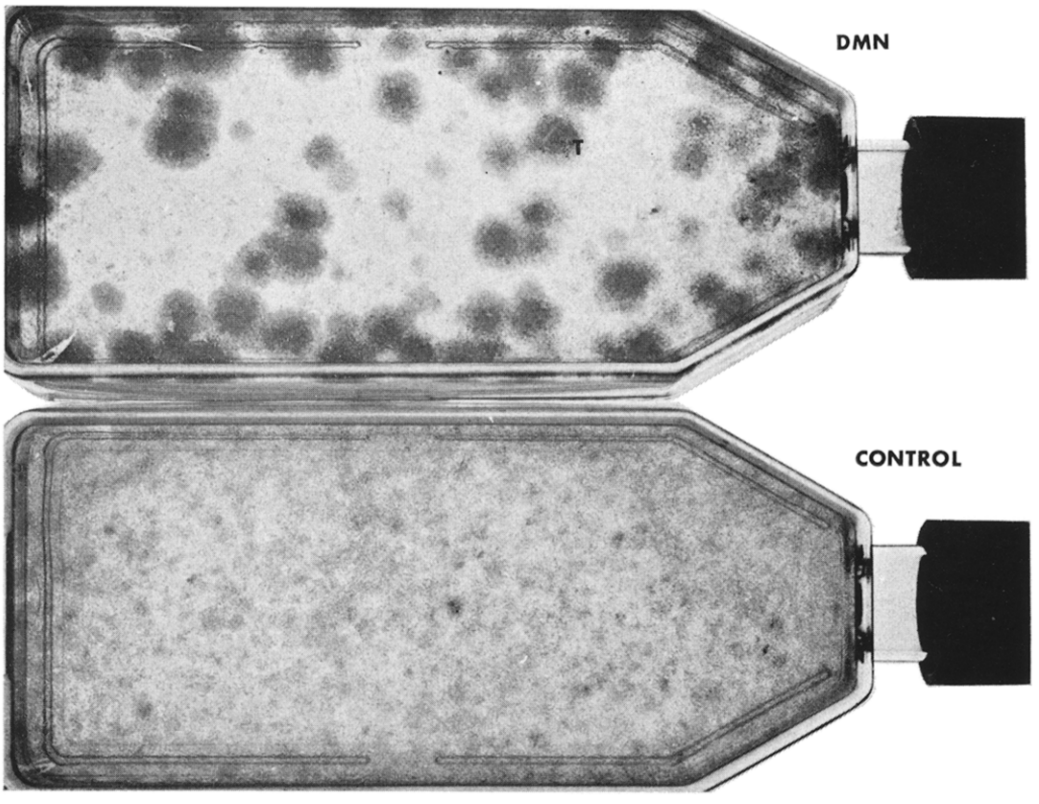


Fig. 2. *Transformed colonies. Subcultures (fourth) of rat kidney cell monolayers from a DMN-treated and a control rat from Experiment B.7 (Fig.* 1). *The transformed colonies (T) are seen as dark circumscribed areas on the photograph. Photographed as haematoxylin and eosin-stained, unmounted cultures* (×2·25).

(to face p. 180)

Table 2. Mitotic counts

Culture type	Cells and experimental numbers	Seeding rate/dish	L.P. fields counted	Number of observed mitoses	Mean No. mitoses/ field
Under 0·6 Agar	Control B7	2×10^5 Cells	40	3	0·1
	Control B9	2×10^5 Cells	40	36	0·9
	DMN B7	2×10^5 Cells	40	110	2·8
	DMN B9	2×10^5 Cells	40	232	5·8
Free Monolayer	Control B12 (Primary)	1×10^5 Cells	40	48	1·2
	DMN B12 (Primary)	1×10^5 Cells	40	32	0·8
	Control B12 (Subculture 2)	1×10^5 Cells	40	10	0·3
	DMN B12 (Subculture 2)	1×10^5 Cells	40	101	2·5

Mitotic figures were counted in 20 low-power (100 × magnification) microscope fields from each of two replicate culture dishes. For experiments numbered DMN B7 and B9, the cultures were examined post-transformation and for experiment B12, the cultures were examined as primary isolates and as early pre-transformation subcultures.

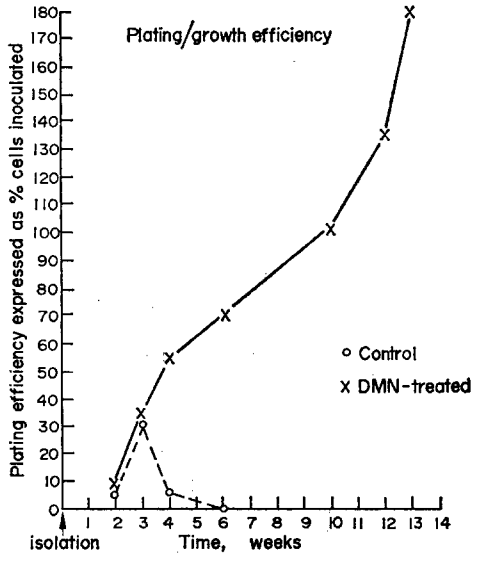


Fig. 3. Plating/growth efficiency. Monolayer cultures of kidneys cells from 3 DMN-treated and 3 control rats were used. Sixteen replicates were seeded for each culture at every time point and after 7 days incubation at 37°C, the surviving cells were fixed in methanol (70%), stained with Giemsa and counted. Plating/growth efficiency is presented as the mean number of surviving healthy cells expressed as a percentage of the initial inoculum.

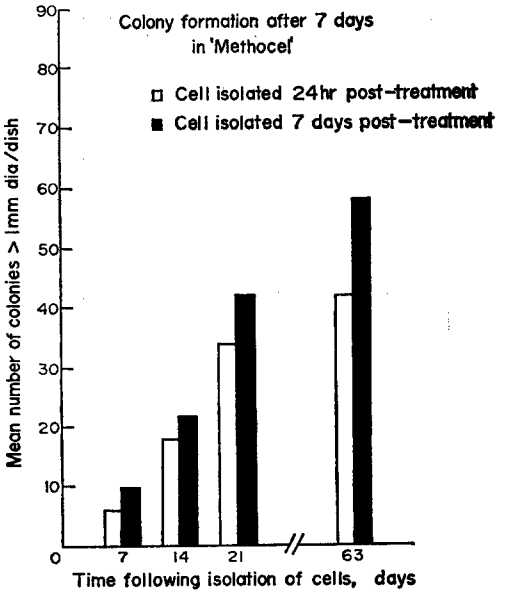


Fig. 4. Colony formation in methyl cellulose. Rat kidney cells cultures were examined from each of 2 treated and 2 control rats at 24 hr and 7 days post-treatment with DMN. After 7 days incubation at 37°C. the cell colonies > 1mm dia were counted in 8 dishes from each culture at each time point and the mean calculated. No colonies were observed with cells from control rats.

presence of Con. A. No agglutination was observed in the suspensions of control cells in the presence of Con. A, but agglutination was observed with all tested cells derived from DMN-treated animals exposed to a Con. A concentration of 100 μg/ml (Table 3). No cyto-agglutination was observed in the absence of Con. A.

DISCUSSION

The kidney cortical cells isolated from rats treated with DMN exhibited a variety of morphological and behavioural changes which indicated that they had transformed. The fact that the changes were observed in cells isolated from animals only 20 hr after treatment suggests that the cells received the transforming stimulus sometime in that period.

Table 3. Cytoagglutination

Cells	Concanavalin A 100 μg/ml	10 μg/ml	Diluent
Control B5	0	0	0
Control B7	0	0	0
Control B9	0	0	0
DMN B5	+ +	0	0
DMN B7	+ + +	+	0
DMN B9	+ +	+	0

Four tests (10^6 cells/test) were carried out on duplicate samples of each culture at each concentration of Con. A. Fewer than 4 cells in an aggregate—0; 4–20 cells in an aggregate—+; 20–40 cells in an aggregate—+ +; more than 40 cells in an aggregate—+ + +.

Prolonged cell growth and an increase in mitotic counts were observed for all the DMN-exposed cells with one exception, whereas the control non-exposed cells showed a very limited duration of *in vitro* growth. Similar increases in cell multiplication and life-span following treatment of hamster cells with high concentrations of DMN, have been reported [21]. The one control cell culture (Fig. 1, B7) which exhibited a prolonged growth pattern developing into what appeared to be a cell line, did not exhibit any of the other transformation characteristics shown by the kidney cells from DMN-treated animals.

The establishment of apparently permanent cell lines with kidney cells from DMN-treated rats is in accordance with the findings in the "direct" *in vitro* treatment of rat cells [22] with other chemical carcinogens and for other animal cells [23–25].

Morphological transformation as characterised by the appearance of colonies showing a random criss-cross distribution of cells was seen, with one exception, in all the cultures derived from the DMN-treated rats. This loss of contact-inhibition of movement [26] and the irregular pattern of the cells in the colonies was similar to that described by other workers using a variety of chemical and viral carcinogens in a number of different cell systems [24,27,28]. The DMN-induced transformation is, however, in direct contrast to the findings of Sanders and Burford [12] who used hamster cells treated *in vitro* with DMN at concentrations of $\leqq 10$ mC ig/ml and failed to produce any evidence of transformation. In a similar study Dipaolo *et al.* [13] using diethylnitrosamine (DEN) and hamster cells failed to produce transformation. However, in a recent study Dipaolo and his co-workers [29] report the induction of transformation in cells from foetal tissue exposed *in utero* to the carcinogen DEN. These findings may be compatible if we assume that DMN and DEN are not "proximate" carcinogens and that they require enzymic conversion to an active intermediate. The cells used by Sanders and Burford [12] and by Di Paolo *et al.* [13] presumably did not possess the necessary enzyme(s) for conversion whereas in the combined *in vivo/in vitro* system described in the present paper, conversion may have taken place either in the kidneys or before the DMN reached the kidneys. With respect to the work of Huberman *et al.* [21], the cells they used may have had the necessary enzymes for DMN-conversion or alternatively the very high concentrations of DMN used may, in some way, have influenced the establishment of a form of "spontaneous" conversion.

The steady increase in the plating/growth efficiency of cells from the DMN-treated rats provides further evidence for the early and continued development of the persistent growth capability of these cells which is consistent with malignant potential. Using another carcinogenic nitroso-compound, methylnitrosourea, Frei and Oliver [30] have reported early enhancement of plating efficiency of mouse primary embryo cells.

The ability of transformed cells to form colonies in soft nutrient gels was described by Montagnier and MacPherson [31] who showed that polyoma virus-transformed baby hamster kidney cells had this capability and that it was not shared by normal cells. In a concurrent study using a similar technique, Sanders and Burford [32] demonstrated that ascites tumour cells, from BHK 21 cells transformed by polyoma virus, would *grow* readily in semi-solid agar medium to form small colonies, whereas the original untransformed clone of bilby hamster kidney cells (BHK) failed to do so. Since then many workers have shown that malignant cells and cells transformed by a variety of carcinogenic agents have the same ability to grow and form colonies in soft nutrient gels [33,34]. The cells from DMN-treated rats exhibited the capacity to grow and form colonies in soft gels as early as seven days after isolation, i.e. before the appearance of morphologically transformed colonies in the cultures. More colonies were observed to develop in soft gels with time following isolation of the cells from the treated rats. At no stage did we observe colony formation with cells from untreated rats. It is of interest that the colony-forming ability in nutrient gels was manifest before the appearance of morphological transformation. This would suggest that growth in a soft gel could be a sensitive early measure of transformation potential.

Positive agglutination in the presence of Con. A was observed with all cells from DMN-treated rats but not with cells from untreated rats. Since Aub *et al.* [35,36] demonstrated that a contaminant of wheat germ lipase preparations agglutinated certain tumour cells, but not normal cells, many workers have confirmed these findings for a wide variety of tumour and transformed cells [37,38]. The agglutinating molecule has been purified and characterised as a plant lectin [39]. Thus it would appear that the kidney cells isolated from DMN-treated rats behave in a similar manner to other tumour and transformed cells when tested for agglutinability in the presence of the plant lectin, Con. A.

The prolonged life span, morphological transformation, increased mitotic index, colony formation in methyl cellulose gels, high relative plating efficiency and Con. A agglutination of kidney cells grown *in vitro* from DMN-treated rats all suggest that these cells are manifestly different from normal and have been transformed. Similar behaviour patterns have been observed with cells established *in vitro* from DMN-induced rat kidney mesenchymal tumours (unpublished observations). Furthermore, cells isolated *in vitro* as early as 20 hr after DMN-treatment of the animal have exhibited the same behaviour patterns as cells removed after seven days. These findings suggest that less than 20 hr *in vivo* exposure to a single dose of DMN will result in alterations in cell growth and behaviour of kidney cells which are consistent with malignant transformation.

The system described above in which cells are isolated *in vitro* from the target organ of an animal exposed *in vivo* to a single carcinogenic dose of DMN could provide an extremely valuable model for the study of the earliest stages of carcinogenesis at both cellular and and subcellular levels.

Experiments are in progress involving syngeneic, allogeneic and xenogeneic transplants of kidneys cells from untreated and DMN-treated rats in addition to a study of cells isolated within the first 20 hr following treatment.

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REFERENCES

1. P. N. Magee and J. M. Barnes, Production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine, *Brit. J. Cancer* **10,** 114 (1956).
2. H. X. Druckrey, D. Steinhoff, R. Preussmann and S. Ivankovic, Krebserzeugung durch einmalige Dosis non Methylnitrosoharnstoss und Verschiedenen Dialkze-nitrosaminen, *Naturwissenschaften* **50,** 735 (1963).
3. P. N. Magee, Alkylation of Nucleic acids and carcinogenesis, 17. *Colloguium der Gesellschaft fur physiologische Chemie,* Mosbach/Springer-Verlag Baden (1966).
4. W. Lijinsky, Evelyn Conrad and Rosalie van de Bogart, Carcinogenic nitrosamines formed by drug/nitrite interactions, *Nature (Lond.)* **239,** 165 (1972).
5. W. Fiddler, J. W. Pensabene, R. C. Doerr and A. E. Wasserman, Formation of N-nitrosodimethylamine from naturally occurring quaternary ammonium compounds and tertiary amines, *Nature (Lond.)* **236,** 307 (1972).
6. J. W. Rhoades and D. E. Johnson, N-dimethylnitrosamine in tobacco smoke condensate, *Nature (Lond.)* **236,** 307 (1972).
7. A. E. McLean and P. N. Magee, Increased renal carcinogenesis by dimethylnitrosamine in protein deficient rats, *Brit. J. exp. Pathol.* **51,** 587 (1970).
8. G. C. Hard and W. H. Butler, Cellular analysis of renal neoplasia: Induction of renal tumours in dietary-conditioned rats by dimethylnitrosamine, with a reappraisal of morphological characteristics. *Cancer Res.* **30,** 2796 (1970).
9. P. F. Swann and A. E. McLean, Cellular injury and carcinogenesis; the effect of a protein-free high-carbohydrate diet on the metabolism of dimethylnitrosamine in the rat, *Biochem. J.* **124,** 283 (1971).
10. P. N. Magee, Toxic liver injury; the metabolism of dimethylnitrosamine, *Biochem. J.* **64,** 676 (1956).
11. R. Schoental, Effects of N- methyl-N-nitrosourethane on cells in tissue culture, *Nature (Lond.)* **215,** 535 (1967).

12. F. K. SANDERS and B. O. BURFORD, Morphological conversion of cells *in vitro* by N-nitrosomethylurea, *Nature (Lond.)* **213,** 1171 (1967).
13. J. A. DI PAOLO, R. L. NELSON and P. J. DONOVAN, *In vitro* transformation of Syrian hamster embryo cells by diverse chemical carcinogens, *Nature (Lond.)* **235,** 278 (1972).
14. A. H. DUTTON and D. F. HEATH, Demethylation of dimethylnitrosamine in rats and mice, *Nature (Lond.)* **178,** 644 (1956).
15. P. N. MAGEE and M. VANDEKAR, Toxic liver injury; the metabolism of dimethylnitrosamine *in vitro*, *Biochem. J.* **70,** 600 (1958).
16. J. A. BROUWERS and P. EMMELOT, Microsomal N-demethylation and the effect of the hepatic carcinogen dimethylnitrosamine on amino acid incorporation into the proteins of rat livers and hepatomas, *Exp. Cell Res.* **19,** 467 (1960).
17. G. C. HARD, R. BORLAND and W. H. BUTLER, Altered morphology and behaviour of kidney fibroblasts *in vitro*, following *in vivo* treatment of rats with a carcinogenic dose of dimethylnitrosamine. *Experientia* **27,** 1208 (1971).
18. K. K. SANFORD, Malignant transformation of cells *in vitro*. *Int. Rev. Cytol.* **18,** 249 (1965).
19. K. K. SANFORD, Spontaneous neoplastic transformation of cells in *in vitro:* some facts and theories, *Nat. Cancer Inst. Monogr.* **26,** 387 (1967).
20. F. K. SANDERS and JANET D. SMITH, Effect of collagen and acid polysaccharides on the growth of BHK/21 cells in semi-solid media. *Nature (Lond.)* **227,** 513 (1970).
21. E. HUBERMAN, S. SALZBERG and L. SACHS, The *in vitro* induction of an increase in cell multiplication and cellular life-span by the water-soluble carcinogen dimethylnitrosamine. *P.N.A.S.* **59,** 77 (1968).
22. H. KATSUTA and T. TAKAOKA, Carcinogenesis in tissue culture. XIV. Malignant transformation of rat liver parenchymal cells treated with 4-nitroquinoline 1-oxide in tissue culture. *J. nat. Cancer Inst.* **49,** 1563 (1972).
23. Y. BERWALD and L. SACHS, *In vitro* transformation of normal cells to tumour cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1965).
24. J. A. DI PAOLO and P. J. DONOVAN, Properties of Syrian hamster cells transformed in the presence of carcinogenic hydrocarbons. *Exp. Cell Res.* **48,** 361 (1967).
25. T. T. CHEN and C. HEIDELBERGER, *In vitro* malignant transformation of cells derived from mouse prostate in the presence of 3-methylcholanthrene. *J. nat. Cancer Inst.* **42,** 915 (1969).
26. M. ABERCROMBIE and E. J. AMBROSE, The surface properties of cancer cells; A review. *Cancer Res.* **22,** 525 (1962).
27. M. NAMBA, H. MASUJI and T. SATO, Carcinogenesis in Tissue Culture. IX. Malignant transformation of cultured rat cells treated with 4-nitroquinoline-1-oxide. *Jap. J. exp. Med.* **39,** 253 (1969).
28. M. STOKER and I. MACPHERSON, Studies on transformation of hamster cells by polyoma virus *in vitro*. *Virology* **14,** 359 (1961).
29. J. A. DI PAOLO, R. L. NELSON and P. J. DONOVAN, Host-mediated *in vivo–in vitro* assay for chemical carcinogenesis. *Arch. Pathol.* **95,** 380 (1973).
30. J. V. FREI and JEAN OLIVER, Early enhancement of plating efficiency of primary mouse embryo cells by the carcinogen methylnitrosurea. *Cancer Res.* **32,** 2747 (1972).
31. L. MONTAGNIER and I. MACPHERSON, Croissance selective en gelose de cellules de Hamster transformees par le virus du polyoma. *C.r. Acad. Sci. Paris* **258,** 4171 (1964).
32. F. K. SANDERS and BARBARA O. BURFORD, Ascites tumours from BHK 21 cells transformed *in vitro* by polyoma virus. *Nature (Lond.)* **201,** 786 (1964).
33. T. R. BRADLEY and D. METCALF, The growth of mouse bone marrow cells *in vitro*. *Austral. J. exp. Biol. med. Sci* **44,** 287 (1966).
34. L. J. ALFRED, Chemical carcinogen-induced alterations in the potentials of cultured animal cells. *Nature (Lond.)* **214,** 732 (1967).
35. J. C. AUB, C. TIESLAU and ANN LANKESTER, Reaction of normal and tumour cell surfaces to enzymes, I. wheat-germ lipase and associated mucopoles saccharides. *Proc. nat. Acad. Sci. U.S.A.* **50,** 613 (1963).
36. J. C. AUB, B. H. SANFORD and M. N. COTE, Studies on reactivity of tumour and normal cells to a wheat-germ agglutinin. *Proc. nat. Acad. Sci. (Wash.)* **54,** 396 (1965).

37. M. Inbar and L. Sachs, Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc. nat. Acad. Sci.* (*Wash.*) **63,** 1418 (1969).
38. L. Mallucci, Binding of concanavalin A to normal and transformed cells as detected by immunofluorescence. *Nature, New Biol.* **233,** 241 (1971).
39. M. M. Burger and A. B. Goldberg, Identification of a tumour-specific determinant on neoplastic cell surfaces. *Proc. nat. Acad. Sci.* (*Wash.*) **57,** 359 (1967).

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Changes in Cell Mediated Immune Functions by Growing Tumor

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Abstract—*Syngeneic strain* 2 *guinea pigs and hepatoma line* 1 *were used to study some features of tumor–host relationship. Three hundred thousand tumor cells were given intramuscularly and* 5 *days later intradermal immunization with living tumor cells was started. At specific time intervals the antitumor response was demonstrated by measuring tumor growth, delayed hypersensitivity reactions at the site of intradermal immunization,* in vitro *stimulation of peritoneal exudate cells (PEC) with tumor cells and* in vitro *PEC-mediated cytoxicity.*

It was found that animals, developing larger tumors than 1 *cm in dia had their imumune response abolished. The specificity of the phenomenon was checked by the ability of the spleen of such animals to produce plaque-forming-cells (PFC) against sheep red blood cells (SRBC).*

The results are discussed in the light of current hypothesis on tumor mediated immunodepression.

INTRODUCTION

THE EVOLUTION of the immune response during tumor growth has been the subject of several recent investigations. At the begin of tumor growth, specific antitumor response both at the humoral and cellular level can be detected in most systems [1,2]. As the tumor keeps on growing, this specific response rapidly decreases, [3] probably because of an unbalance in the defense mechanism, brought about by some kind of blocking factors [2,4,5]. However, at this stage, general immune reactivity to antigens other than the tumor carries would exist [2,6], although the occurrence of nonspecific immunosuppressive agents has also been described [7,8]. Finally, when the tumor becomes large and the malignant disease widespread, the immune responsiveness is often impaired in a nonspecific manner [9].

In our present study we confirm some of these observations and evaluate the possible effects of immunotherapy upon cell mediated immune functions, when given during the latent period of tumor growth. Three recognized paramaters of cell mediated immunity have been measured in parallel: blastogenic transformation and immune cytolysis by PEC *in vitro* and delayed skin reactivity *in vivo*. Although these parameters measure different events of the cellular immune response, it could be shown that they were changed in the same way by the presence of the tumor.

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MATERIAL AND METHODS

Animals and tumors

Four-to-six-month-old female Sewall–Wright strain 2 guinea pigs were used. Between 2–5 guinea pigs were kept in each plastic cage and fed with fresh salad and carrots. Hepatoma line 1, a transplantable adenocarcinoma, induced and described by Rapp and coworkers [10–12], was kept either *in vivo* by serial transplantation or stored with 10% DMSO in liquid nitrogen. A tissue culture line of this tumor has been successfully established by one of us and checked by chromosome analysis.

Immunization

Small solid tumor pieces were prepared in a salt solution containing glucose, potassium and sodium and digested for 90 min with 0·1% collagenase (B-grade, Calbiochem) to yield a

single cell suspension. If dead cells were present, they were removed by centrifugation on a BSA-gradient as described by Wepsic [13]. The washed tumor cells were suspended in PBS at 15×10^6/ml and 0·1 ml were injected intradermally at two sites on the animals white back [12]. Skin reactions were read at 24 hr and 48 hr and scored as −(0–4 mm), +(5–9 mm), ++(10–14 mm), +++(15–19 mm), ++++(20 mm and more). Animals were used as "immune" after three such immunizations.

In vitro *tests*

1. Plaque-forming cell assay: SRBC were purchased from Serotherapeutisches Institut, Vienna, washed three times with saline and 2 ml of a 20% suspension was injected by the intracardiac route. 5 days later the spleen was removed and PFC enumerated according to the method of Cunningham and Szenberg [14]

2. Mixed-PEC tumor cell reaction: PEC from immune or normal animals were collected 4–7 days after an intraperitoneal injection of 12 ml paraffin oil (USP XVII, Merck), washed three times and suspended to 2×10^6/ml in RPMI 1640 with 10% Fetal Bovine Serum (FBS) or Human AB-Serum. The suspension contained 70–80% Macrophages, 12–20% Lymphocytes and 5–15% Granulocytes as determined by Giemsa staining. Hepatoma line 1 cells were treated with 50 μg/ml of Mitomycin C (Sigma Biochemicals) for 30 min, washed three times and suspended to 1×10^5/ml. Triplicate cultures were set up by mixing 1 ml of PEC either with 1 ml of stimulating tumor cells or with 1 ml of medium. These were then incubated for three days in a moist atmosphere containing 5% CO_2 and pulsed overnight with 1 μCi of H3-Thymidine (5 Ci/mmol, Radiochemical Center Amersham). On the following day material precipitable by acid was processed for liquid scintillation counting. A typical kinetic response curve is given in Fig. 1.

3. Cell mediated cytotoxicity tests were done as described by MacPherson and Pilch [15], measuring remaining H3-Thymidine activity in prelabeled tissue culture target cells after 48 hr exposure to PEC at a ratio of 100:1. Cytotoxic index has been expressed as the quotient of the activity remaining after treatment:

$$\frac{\text{Immune PEC–Normal PEC}}{\text{Normal PEC}}$$

Statistical analysis of results has been done by Student's *t*-test.

RESULTS

Groups of five animals each received either 300,000 tumor cells alone intramuscularly or tumor plus 4 intradermal injections of living hepatoma line 1 starting five days later. Two other groups of animals received only the intradermal immunization course or were left wholly untreated. Weekly measurements of tumor diameter revealed either enhanced or depressed tumor growth as compared to controls receiving no immunization (Fig. 2). This points to a dual effect of immunization in this system, either promoting or inhibiting tumor growth. By testing *in vitro* each animal at the indicated time

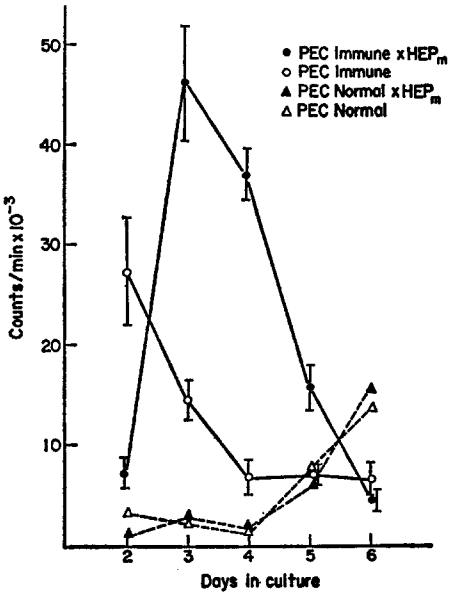


Fig. 1. Kinetic response of immune and normal PEC in mixed cultures with Mitomycin C treated hepatoma line 1. Each point represents the arithmetic mean of triplicate cultures. Vertical bars indicate the standard error of mean.

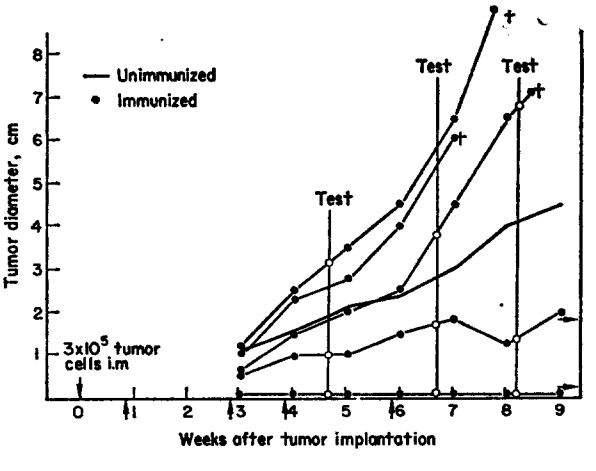


Fig. 2. Tumor growth of hepatoma line 1 in immunized and unimmunized strain 2 guinea pigs. The heavy line depicts the mean tumor growth in seven control animals. Intradermal immunization with 3×10^6 tumor cells (indicated by arrows) was started 5 days after tumor implantation.

we found that the capability of specific cell mediated immune response correlates negatively with the tumor burden with which the bodies immune system is faced (Fig. 3 and 4): The animal could respond if the tumor was very small. However, as the tumor mass exceeds 1 cm in dia, the immune response strikingly decreases and turns even to negative. A similar inhibition could also be detected by delayed skin reactions. In contrast, no special decline in reactivity could be seen with the PFC response, indicating that general immune reactivity was retained even in otherwise unreactive animals.

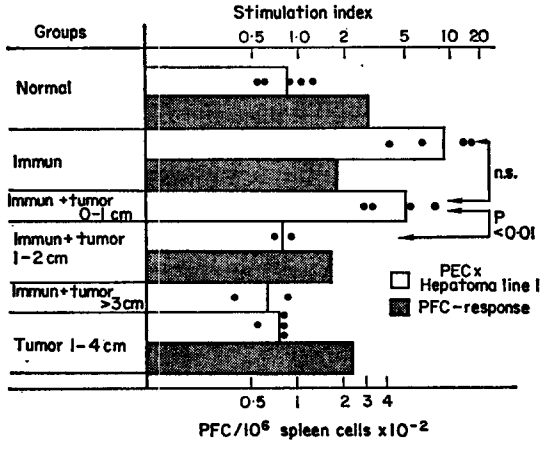


Fig. 3. Stimulation of PEC from immune and tumor bearing guinea pigs by hepatoma line 1, and PFC-response against SRBC. Each point represents the arithmetic mean of triplicate cultures.

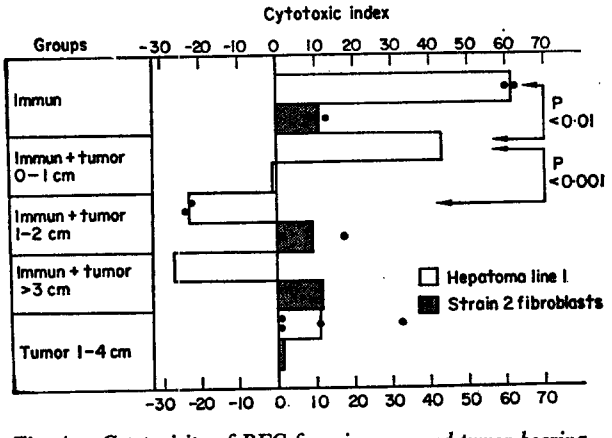


Fig. 4. Cytotoxicity of PEC from immune and tumor bearing guinea pigs against hepatoma line 1 and strain 2 fibroblasts. Each point represents the arithmetic mean of triplicate cultures.

DISCUSSION

From the above experiments we conclude that in our system a growing tumor, when exceeding the small size of 1 cm, is able to abolish specifically a developing immune response against it. Similar findings have been reported recently by Youn *et al.* [6], using a mouse tumor system. They noted an eclipse of the animals immune response when tumors rose above 10 mm in dia. This could be partially ascribed to blocking serum factors which were acting primarily upon the effector cells. Although a more detailed search for responsible serum factors would be necessary in our system, these could not alone explain the phenomenon, because all cell preparations have been washed three times before testing and cultures were set up in neutral serum.

Several arguments can be raised to explain the tumor mediated inhibition. (1) The tumor could deviate the immune response by the induction of IgG 1 antibody, which in turn inhibits the formation of the more cytophilic and cytotoxic IgG 2 antibodies otherwise produced by immunization through the intradermal route [16]. Immunodeviation may be one of the reasons why immunization after tumor grafting is much less effective against the tumor than prophylactic immunization. (2) Another possibility is the production of a factor by the tumor which directly affects the host immune response. Evidence for such a mechanism of inhibition has been found both in humans [17] and animals [18]. Most likely this factor would be antigen or antigen–antibody complexes released by the tumor. (3) Prehn has proposed that an antitumor response could actually stimulate tumor growth, especially if the tumor antigens are weak [19,20] This has been explained by the more important selection pressure for the survival of the fetus in mammals where a weak immune response of the mother is favourable for his development. Such an argument may also explain why we found in 3 out of 5 immunized animals both *in vitro* and *in vivo* an enhancement of tumor growth above the level of unimmunized animals

Our findings are however in apparent contrast with results from Simmons and coworkers who achieved successful immunotherapy in mice carrying established tumors [21]. In their experiments, pretreatment of the cells with neuraminidase and the use of BCG may have been critical and perhaps these are possible ways of reversing the effects of the tumor on the immune response. Despite these encouraging results, one should bear in mind, when dealing with a patient's immune response, that the tumor may have had already an effect on the patients immune response.

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REFERENCES

1. G. Barski, J. K. Youn, Evolution of cell mediated immunity in mice bearing an antigenic tumor. Influence of tumor growth, surgical removal and treatment with irradiated tumor cells. *J. nat. Cancer Inst.* **43,** 111 (1969).
2. A. M. Cohen, R. C. Millar and A. S. Ketcham, Host immunity to a growing transplanted MCA-induced guinea pig sarcoma. *Cancer Res.* **32,** 2421 (1972).
3. Z. B. Mikulska, C. Smith and P. Alexander, Evidence for immunological reactivity of the host directed against its own actively growing primary tumor. *J. nat. Cancer Inst.* **36,** 29 (1966).
4. R. W. Baldwin, M. R. Price and R. A. Robins, Blocking of lymphocyte mediated cytotoxicity for rat hepatoma cells by tumour specific antigen-antibody complexes. *Nature, New Biol.* **238,** 185 (1972).
5. I. Hellström, H. O. Sjögren, G. Warner, K. E. Hellström, Blocking of cell mediated tumor immunity by sera from patients with growing neoplasma. *Int. J. Cancer,* **7,** 226 (1971).
6. J. K. Youn, D. LeFrancois and G. Barski, *In vitro* studies on mechanism of the "eclipse" of cell mediated immunity in mice bearing advanced tumors. *J. nat. Cancer Inst.* **50,** 92 (1973).
7. K. Ashikawa, K. Inoue, T. Shimizu and Y. Ishibashi, An increase of serum alpha-globulin in tumor-bearing hosts and its immunological significance. *Japan. J. exp. Med.* **41,** 335 (1971).
8. R. W. Gillette, Ch. W. Boone, Changes in PHA-response due to presence of tumors. *J. nat. Cancer Inst.* **50,** 1391 (1973).
9. G. F. Rowland, A. J. Edwards, M. R. Sumner and C. M. Hurd, Thymic dependency of tumor induced immunodepression. *J. nat. Cancer Inst.* **50,** 1329 (1973).
10. H. J. Rapp, W. H. Churchill, Jr., B. S. Kronman, R. T. Rolley, W. G. Hammond, T. Borsos, Antigenicity of a new diethylnitrosamine induced transplantable guinea pig hepatoma: pathology and formation of ascites variant. *J. nat. Cancer Inst.* **41,** 1 (1968).
11. B. Zbar, H. T. Wepsic, H. J. Rapp, T. Borsos, B. S. Kronman, W. H. Churchill, Jr., Antigenic specificity of hepatoma induced in strain 2 guinea-pigs by diethylnitrosamine. *J. nat. Cancer Inst.* **43,** 833 (1969).
12. W. H. Churchill, Jr., H. J. Rapp, B. S. Kronman, T. Borsos, Detection of antigens of a new diethylnitrosamine-induced transplantable hepatoma by delayed hypersensitivity. *J. nat. Cancer Inst.* **41,** 13 (1968).
13. H. T. Wepsic, Separation of viable tumor cells from non viable tumor cells by flotation on bovine serum albumin. A short communication. *J. nat. Cancer Inst.* **45,** 1031 (1970).
14. A. J. Cunningham, A. Szenberg, Further improvements of the plaque forming cell technique for detecting single antibody forming cells. *Immunology* **14,** 599 (1968).
15. B. R. MacPherson and Y. H. Pilch, Cellular cytolysis *in vitro:* mechanism underlying a quantitative assay for cellular immunity. *J. nat. Cancer Inst.* **48,** 1619 (1972).
16. P. Vuagnat, Th. Neveu and G. A. Voisin, Immunodeviation by passive antibody, an expression of selective immunodepression. *Europ. J. Immunol.* **3,** 90 (1973).
17. A. J. Edwards, G. F. Rowland, M. R. Lee, Reduction of lymphocyte transformation by a factor produced by gastrointestinal cancer. *Lancet,* **i**678. (1973).
18. D. B. Amos, I. Cohen and W. J. Klein, Jr., Mechanisms of immunologic enhancement. *Transpl. Proc.* **2,** 68. (1970).
19. R. T. Prehn, Prospectives in oncogenesis: does immunity stimulate or inhibit neoplasia? *J. reticuloendothel. Soc.* **10,** 1 (1971).
20. I. J. Fidler, *In vitro* studies of cellular mediated immunostimulation of tumor growth. *J. nat. Cancer Inst.* **50,** 1307 (1973).
21. R. L. Simmons and A. Rios, Immunotherapy of cancer: immunospecific rejection of tumors in recipients of neuraminidase treated tumor cells plus BCG. *Science (N.Y.)* **174,** 591 (1971).

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Interferon Production by Leukocytes Cultures from Neoplastic Patients

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Abstract—*Interferon induction by leukocytes from* 33 *patients with solid tumours of different localization was* 2 *to* 16 *times lower than by leukocytes obtained from healthy donors, or from non-neoplastic patients with serious illnesses.*

THE PRODUCTION of Interferon by suspensions of human leukocytes has been previously demonstrated. [1–4].

The ability to produce Interferon is modified in acute and chronic leukemia patients. Lee *et al.* [5, 6] established that leukocytes collected from acute lymphocytic, acute myelogenous and long-term chronic lymphocytic leukemia patients under treatment had an impaired capacity to produce Interferon, while normal levels were found to be produced by leukocytes from recently diagnosed or recently treated, chronic lymphocytic leukemia patients. In some cases an enhanced Interferon response was observed in leukocytes from chronic myelogenous leukemia patients.

Veskova [7] observed that leukocytes from Hodgkin disease patients produce 10–20 times less Interferon than leukocytes from normal donors. These values were lower than those reported for acute myelogenous leukemia.

Considering that patients with cancer sometimes have a depression of their immunological response and a marked sensitivity to viral illnesses, we thought it would be interesting to investigate whether these patients have also a reduced Interferon response.

The present work consists of an analysis of Interferon production by leukocyte cultures from untreated neoplastic patients, carriers of solid tumours, compared with production by cells of non-neoplastic patients and normal donors.

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MATERIAL AND METHODS

Cells

(a) *Leukocyte culture from human peripheral blood.* Twenty-five ml of venous blood was gently mixed with a sodium-citrate–glucose anticoagulant solution and used within 2 hr. Leukocytes were separated by adding 1 part of a 6% Dextran solution (MW: 180.000) to 4 parts of blood; the mixture was kept at 4°C for 1 hr and was shaken gently a few times. The upper layer, containing the leukocytes, was removed and centrifuged for 10 min at 1000 rev/min. The pellet was washed once with phosphate buffer pH 7. The number of cells per culture was determined by hemocytometer count. The viability of lymphocytes was determined by the trypan blue exclusion test. Cultures were carried in 50 ml Erlenmeyer with Parker's 199 medium supplemented with 2·5% fetal calf serum. The cell concentration used for Interferon induction was 5×10^6/ml.

(b) *FS cells diploid human cell line.* (Foreskin Wistar Institute, Philadelphia) were maintained in our laboratory in Eagle's MEM supplemented with 10% calf serum.

Virus

Stock Sendai virus (Parainfluenza type I) used to induce and to titrate Interferon was maintained in our laboratory by passage in allantoic fluid of 9 days chick embryos. Allantoic fluid was collected after 72 hr incubation at 36°C; it was clarified by centrifugation, stored in 1 ml vials and kept at −100°C. The chick erythrocyte hemagglutination titer was of 1/1024.

Interferon titration. Interferon was determined by incubating duplicate monolayers of FS cultures in Leighton tubes with two-fold dilutions in MEM containing 2% calf serum, for 20 hr. After washing with 2 ml of culture medium, 0·1 ml of a 1/10 dilution of Sendai virus was added to each tube. After 2 hr of incubation at 37°C the virus inoculum was discarded, and cultures were again washed with 2 ml of medium; 1 ml of fresh medium was added to each tube which was incubated for 24 hr more at 37°C (time required for one complete cycle of virus replication) and then frozen at −40C°.

The virus harvested from duplicate monolayers was pooled, clarified by centrifugation at 1000 rev/min for 5 min and titrated by hemagglutination reaction.

Interferon titers were expressed as the reciprocal of the dilution which reduced the hemagglutinin titer by $0{\cdot}5 \log_{10}$. Control Interferon titer was of 2048 Units/ml.

Statistical analysis. Data were analyzed according to *t*-test on logarithmic titers of the induced Interferon.

RESULTS

Interferon induction

A leukocyte suspension in phosphate buffer was infected with Sendai virus at a multiplicity of infection of 10.

After 2 hr at 37°C, non-adsorbed virus was discarded by centrifugation and cells were washed with Alsever solution containing 10% normal human serum. The pellet was resuspended in medium 199 with 2·5% fetal calf serum and then incubated for 24 hr at 37°C. The cultures were centrifuged and the supernatant fluid containing Interferon was characterized and titrated for Interferon content

Interferon production from cell cultures prepared from each individual patient was characterized as follows: dialysis at pH 2 during 48 hr followed by neutralization with 1N NaOH and ultracentrifugation at 100,000 *g* for 2 hr. After this treatment samples were filtrated through Sartorius filters (pore size 0·22μ), stored in siliconized flasks and kept at −100°C. Each sample was given a code number so the titration assays were made blind.

The same method of induction was followed to prepare a control reference interferon from a pool of blood of 20 healthy donors. Besides the characterization reactions already described, it was verified that the control Interferon was inactivated by heating for 1 hr at 56°C and by trypsin digestion. The molecular weight was estimated by chromatography in Sephadex gel G 100 and was about 25,000 daltons. Synthesis of this control interferon was inhibited with 1 μg/ml of Actinomycin D.

Patients

Thirty-seven patients without any previous treatment were selected for our study. Thirty-three of them were neoplastic patients being generally in an advanced stage of their disease.

None of them were suffering from any clinically detectable viral disease at the moment of bleeding.

Interferon titers obtained from leukocyte cultures of neoplastic patients with several histopathological types and different tumour sites are shown in Table 1. The ability to produce Interferon by leukocytes from neoplastic patients was 2–16 times lower than from healthy donors.

Table 2 shows that the average interferon titers induced in cells from neoplastic patients was 4·3 times lower than in those of healthy donors or non-neoplastic patients.

Decrease of the Interferon titers induced in neoplastic patients was found to be statistically significant when compared with values of control donors at a *P*-level $< 0{\cdot}01$. The lowest titers were obtained in patients with metastasic or with undifferentiated infiltrating tumours.

It is noteworthy that one of the patients who received an immunostimulation with BCG increased two times his ability to produce Interferon. It is interesting that a titer of Interferon four times lower than normal was also found in a patient suffering erythematous lupus, an autoimmune disease.

DISCUSSION

Although the number of cases is not very important, these experiments seem to show that Interferon induction from leukocytes from 33 patients with solid tumours at different localizations was 2–16 times lower than those obtained from healthy donors or from non-neoplastic patients with serious illnesses. The Interferon titers obtained from normal donors were similar to those reported by other authors.

As has been found in normal healthy donors by Ray [8], no correlation existed between Interferon titers and patient's age or sex, values being generally low in every neoplastic case. In a non-neoplastic disease as is polycythaemia vera, Hadházy *et al.* [9] reported a 6-fold lower Interferon production.

Table 1. *Comparison of amount of Interferon induced in human leukocytes cultures from neoplastic and non-neoplastic patients and normal donors*

Tumor site	Histopathology	No. of patients	Average Interferon titer (units/ml)
Mammary gland	adenocarcinoma	5	512
Mammary gland	carcinoma	6	362·6
Lung	squamous cell carcinoma	6	384
Thyroid	carcinoma	2	768
Leg	melanoblastoma	1	265
Larynx	semidifferentiated squamous cell carcinoma	1	128
Esophagous	adenocarcinoma	2	384
Mediastinal syndrome	metastasis of undifferentiated carcinoma	2	384
Rectum	adenocarcinoma	1	512
Lymph node	fibrolymphosarcoma	1	1024
Ilium	chondrosarcoma	1	1024
Leg	synovial sarcoma	1	256
Groin	fibrosarcoma	1	512
Ovary	carcinoma	1	256
Generalized myeloma	plasmocytoma	1	512
Pancreas	—	1	256
Non-neoplastic disease:			
Gastric ulcus		2	2048
Peritoneal tuberculosis		1	2048
Erythematous lupus		1	512
Normal donors		20	2048

Table 2. *Mean number of Interferon values in neoplastic, non-neoplastic and normal donors*

Patients	No. of cases	Average Interferon titer (units/ml)
Tumor-carriers	33	476·2
Non-neoplastic disease*	3	2048
Normal donors	20	2048

*Erythematous lupus case was excluded because it is an autoimmune disease.

Our results cannot be compared with those of Veskova [7] in Hodgkin's disease and Lee's [5, 6] in leukemia because the patients selected by them had received chemotherapeutic or radiotherapeutic treatment which modify the immunological response. However in a comparative study on the Interferon production by the leukocytes of healthy and leukaemic subjects, Hadházy *et al.* [10] observed 15 times more Interferon from leukocytes of patients with myeloid leukemia than those of controls.

Studies on acute leukemia and infections mononucleosis of childhood [11] indicated that lymphoblastoid cells derived from transformed bone marrow cultures of patients with leukemia or infections mononucleosis produced Interferon spontaneously in the absence of human cell monolayers, whereas peripheral blood leukocytes did not.

Our results agree with Veskova's [7]; in two cases of untreated solid malignant tumours were titers of Interferon 2–5 times lower than normal ones.

In conclusion, the probable immunodepression of the cancer patients (evidence that is not available for our patients because the clinical immunological status was not evaluated) was paralleled by a lower ability to produce Interferon, at least in advanced stages of the disease. Several explanations for this observation are possible.

Serum blocking factors of cancer patients might hinder leukocytes to receive viral stimulation for Interferon induction.

Alternatively, the inherent ability of lymphocytes or any other white cells to produce Interferon may be blocked or repressed.

Epstein *et al.* [12] suggest that an immunotype "recall" phenomenon function could take place in Interferon response and it could be altered in cancer patients.

REFERENCES

1. I. Gresser, Production of Interferon by suspensions of human leukocytes. *Proc. Soc. exp. Biol. Med.* **108,** 799 (1961).
2. E. Falcoff, R. Falcoff, F. Fournier et C. Chany, Production en masse, purification partielle et caractérisation d'un Interferon destiné a des essais thérapeutiques humains. *Ann. Inst. Pasteur* **111,** 562 (1966).
3. E. F. Wheelock, Virus replication and high-titered Interferon production in human leukocyte cultures inoculated with Newcastle disease virus. *J. Bacteriol.* **92,** 1415 (1966).
4. H. Strander and K. Cantell, Further studies on the production of Interferon by human leukocytes *in vitro*. *Ann. med. exp. Fenn.* **45,** 20 (1967).
5. S. H. S. Lee, R. L. Ozere and C. R. Van Rooyen, Interferon production by human leukocytes *in vitro*. Reduced levels in lymphatic leukemia. *Proc. Soc. exp. Biol. (N.Y.)* **122,** 32 (1966).
6. S. H. S. Lee, C. E. Van Rooyen and R. L. Ozere, Additional studies of Interferon production by human leukemic leukocytes *in vitro*. *Cancer Res.* **29,** 645 (1969).
7. T. K. Veskova and B. M. Nemiroskava, Interferon production by blood leukocytes in Hodgkin's disease. *Rev. Europ. Etudes clin. biol.* **XVI,** 697 (1971).
8. C. G. Ray, The ontogeny of Interferon production by human leukocytes. *J. Pediatr.* **76,** 94 (1970).
9. Gy. Hadházy, L. Gergely, Gy. Nagy and F. D. Toth, Comparison of Interferon production *in vitro* by leukocytes from healthy and polycythaemic persons. *Acta Microbiol. Acad. Sci. Hung.* **15,** 141 (1968).
10. Gy. Hadházy, L. Gergely, F. D. Toth and G. Szegedi, Comparative study on the Interferon production by the leukocytes of healthy and leukaemic subjects. *Acta Microbiol. Acad. Sci. Hung.* **14,** 391 (1967).
11. R. M. McCombs and M. Benyesh-Melnick, Studies on acute leukemia and infectious mononucleosis of childhood. I. Viral interference with lymphoblastoid cells of spontaneously transformed bone marrow. *J. natn. Cancer Inst.* **39,** 1187 (1967).
12. L. B. Epstein, J. Cline and T. Merigan, The interaction of human macrophages and lymphocytes in the Phytohemagglutinin-stimulated production of Interferon. *J. clin. Invest.* **50,** 744 (1971).

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Letter to the Editor

Cytotoxic Effect of 14-Membered Heterocyclic Compounds on HeLa and Human Embryonic Fibroblast Cells

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INVESTIGATION of the cytotoxicity of various natural and synthetic materials using cultured cells is of increasing importance. Despite the limitations of such *in vitro* studies, it seems a feasible method for screening compounds for cytotoxic activity [1–3] or for testing anti-tumour drugs [4, 5].

The results obtained with some new compounds [6] against HeLa and human embryonic fibroblast (HEF) cells are reported. Actinomycin D and Colchicine were also tested as positive controls.

Two methods were employed. In the first, monolayer cultures of HeLa and secondary HEF cells (in medium Parker 199 supplemented with 10% bovine serum, 100 units per ml of Penicillin and 100 mg per ml Streptomycin) were grown in test tubes and treated with serial two-fold dilutions of the compound under test. When the so called critical dose—the highest dilution to damage the cells—was established, a more detailed experiment was carried out. The modified method described by Barranco *et al.* [7] and Li *et al.* [8] was used. Cells grown in Roux bottles were detached by trypsinisation, counted in a haemocytometer and diluted to give 500–1000 cells/ml of the medium containing the appropriate concentration of the substances. The medium was then added in 1 ml aliquots to small bottles of 2 cm^2 of culture space. Bottles were closed with rubber stoppers and incubated at 37°C for 48 hr. One set of the bottles (for parallels of each dilution of the compounds, and five controls) of both HeLa and HEF cells was removed after 24 hr incubation. The medium was then decanted, the cells washed three times with PBS, fixed with methanol and stained by crystal violet. All of the cell colonies remained attached to the glass surface after the treatment were counted by stereo microscopy. The second set of the cells was stained similarly after 48 hr incubation. Cell damage was expressed as a percentage of controls. Usually three experiments were carried out for each critical concentration of a compound.

As Fig. 1 shows, 5 μg/ml of Colchicine inhibits cell growth considerably, although surviving cells could be found after 48 hr of incubation. This illustrates the well known fact that Colchicine is primarily a cytostatic and not a cytotoxic drug. Actinomycin D kills cells extensively, however, 30% of the cells could still attach to the glass surface in the presence of the antibiotic at a concentration of 5 μg/ml, but after 48 hr there was a large-scale reduction (90% cell kill) of viable cells. Colchicine and Actinomycin D killed both HeLa and HEF cells to approximately the same extent.

Some of the most active heterocyclic compounds were selected for cytotoxicity tests on HeLa and HEF cells. Figure 2 summarises the general structure of the compounds tested, and their structure–activity relationships.

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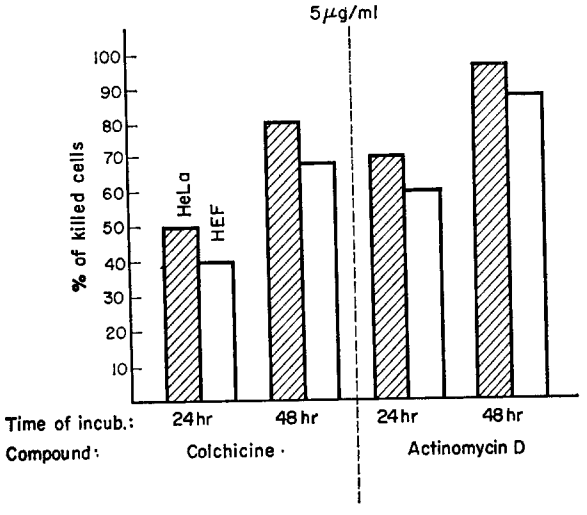


Fig. 1.

Compound HA 1603 (R = 3,4-dimethoxyphenyl) had no observable effect on either HeLa cells or normal HEF cells and the same result was obtained with the Cu complex (HA 81). When R = 2,4-dichlorophenyl (HA 1659), an intensive toxic effect was found which was similar for both types of cells. A more toxic variant of the molecule was achieved when R = phenyl (HA 68) but again there was no difference in the effect on the two cell types. The 4-chlorophenyl derivative (HA 76) killed both HeLa and HEF cells at a concentration of 1 μg/ml. Compound HA 1622 with a 2-naphthyl substituent had not only significant toxicity but its effects on the two cell types differed considerably. At a concentration of 5 μg/ml it killed 60% of the HeLa cells in the first 24 hr but did not affect the fibroblasts. After 48 hr incubation when 100% of the HeLa cells were affected, more than 30% of the HEF cells could still form colonies.

The results presented here are further examples of the usefulness of cell cultures in testing cytotoxic substances. Nevertheless we are aware of the fact that cells cultured *in vitro* may not always respond in the same way as the same cells growing *in vivo*. However, for testing a series of newly synthesised compounds either for cytotoxicity or for viral inhibitory activity [9], *in vitro* techniques represent a convenient method. Furthermore, it gives an excellent opportunity to elucidate the structure-activity relationships of the compound tested.

In our experiments using tumour and normal human cells, some selective toxicity could be determined especially with compound HA 1622 which had a markedly stronger cytotoxic effect on HeLa cells. However this effect is not necessarily a specific "antitumour" or "anti-epithelial" action.

The results presented here show that the toxicity of the compounds depends on the nature of the substituent groups. One compound (where R = 2-naphthyl) was found to have a pronounced cytotoxicity for HeLa cells. None of the other compounds tested nor Colchicine or Actinomycin D had such a selective effect.

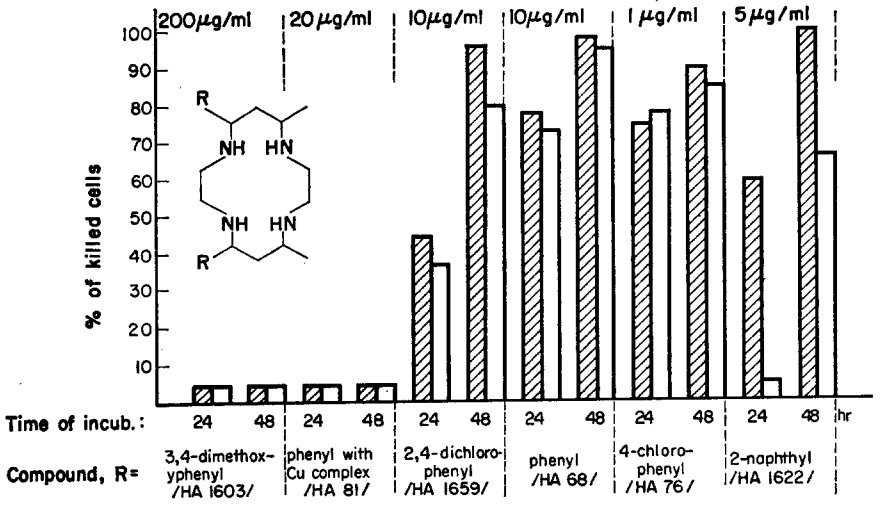


Fig. 2.

REFERENCES

1. E.O.R.T.C. Screening Group, Handbook of materials and methods. *Europ. J. Cancer* **8,** 185 (1972).
2. C. T. Warnick, H. Muzik, A. R. P. Paterson, Interference with nucleoside transport in mouse lymphoma cells proliferating in culture. *Cancer Res.* **32,** 2017 (1972).
3. P. R. Cooper and H. Smith, Influence of cell free ascites fluid and adenosine 3′5′-cyclic monophosphate upon the cell kinetics of Ehrlich's ascites carcinoma. *Nature (Lond.)* **241,** 457 (1973).
4. R. A. Tobey, Effect of cytosine arabinoside, daunomycin, mithramycin, azacytidine, adriamycin, and campothecin on mammalian cell cycle traverse. *Cancer Res.* **32,** 2720 (1972).
5. K. Curtis and F. T. Perkins, Antitumour activity of mithramycin. *Med. Microbiol. Immunol.* **158,** 64 (1972).
6. K. Hideg and D. Lloyd, Reaction products from unsaturated ketones and aliphatic diamines or dithiols. *J. chem. Soc. (C)* 3441 (1971).
7. S. C. Barranco, D. H. W. Ho, B. Drewinko, Differential sensitivities of human melanoma cells grown *in vitro* to Arabinosyl-cytosine. *Cancer Res.* **32,** 2733 (1972).
8. L. H. Li, T. J. Fraser, E. J. Olin and B. K. Bhuyan, Action of Campothecin on mammalian cells in culture. *Cancer Res.* **32,** 2643 (1972).
9. R. H. Adamson, Hydroxyguanidine—a new antitumour drug. *Nature (Lond.)* **236,** 400 (1972).

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Books Received

The receipt of the following books is acknowledged, and this listing must be regarded as sufficient return for the courtesy of the sender.

Tumor Lipids: Biochemistry and Metabolism: Edited by RANDALL WOOD, 250 pp., illustrated. Champaign, Illinois: American Oil Chemists's Press, 1973. Price not indicated.

Tratto di Oncologia Clinica. Volume I: P. BUCALOSSI and UMBERTO VERONESI. 564 pp., illustrated. Milano: Casa Editrice Ambrosiana, 1973. Price not indicated.

Tratto di Oncologia Clinica. Volume II: P. BUCALOSSI and UMBERTO VERONESI. 895 pp., illustrated. Milano: Casa Editrice Ambrosiana, 1973. Price not indicated.

Tratto di Oncologia Clinica. Volume III: P. BUCALOSSI and UMBERTO VERONESI. 1002 pp., illustrated. Milano: Casa Editrice Ambrosiana, 1973. Price not indicated.

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

2nd meeting organized by the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands, October 3–5, 1973, *in the Netherlands Congress Centre, The Hague, The Netherlands*

Introduction to the Proceedings

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

INTEREST in the clinical application of ionizing radiations that deposit energy through particles of high LET, fast neutrons, negative pions and heavy ions, has continued to increase during the last years. This is partly due to the availability of an increasing number of radiation facilities providing beams of adequate intensity and penetration and of new data with respect to radiobiological effects on tissues and tumours in animals. The most important development is, however, that encouraging results have been obtained in the treatment of patients with fast neutrons from the MRC cyclotron at Hammersmith Hospital, London, and, although for much smaller numbers of patients, with fast neutron beams in other centres.

This second meeting was organized to review the progress made since the first meeting held in Rijswijk in 1970, with respect to technical, physical and radiobiological aspects of fast neutron radiotherapy, and to discuss clinical data obtained in several centres with a view of providing guidance for future clinical trials and stimulating international cooperation among all centres involved.

During the first two days of the meeting, sessions were held on sources of fast neutrons, negative pi-mesons and heavy ions, on collimation and depth–dose characteristics of fast neutrons, while other sessions dealt with the relative biological effectiveness of these radiations for the induction of damage to various types of normal tissues and experimental tumours. During the sessions of the third day, clinical experience with fast neutrons from various centres was reported, and plans for future application of fast neutrons, pi-mesons and heavy ions were discussed.

The meeting was attended by 187 participants from 14 countries and was financially supported by the Organization for Health Research TNO, the Ministry of Education and Sciences of the Netherlands, and the Commission of the European Communities (Euratom), Belgium.

Thanks are due to the many individuals who helped in the planning and organization of this meeting, in particular to Mrs. M. W. Th. Purmer-Wagener and Mrs. H. Zandstra-Overvoorde. With respect to the proceedings of this meeting, our thanks are due to Prof. H. J. Tagnon, editor of the European Journal of Cancer who again made it possible to have the manuscripts published in this most efficient way [1].

We hope that the publication of the proceedings will serve as a useful source of information and will contribute to an international

cooperation in this area of clinical radiotherapy, stimulated and guided by results of radiobiological investigations. It is of interest to note that as a sequel to this meeting the E.O.R.T.C. (European Organization for Research on Treatment of Cancer) has decided to sponsor the formation of a Clinical Cooperative Group on Fast Neutron Therapy to further cooperation among active centres in Europe.

REFERENCE

1. The Proceedings of the first meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy were published in *Europ. J. Cancer* **7,** 97–267 (1971).

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Introduction to the Discussion on Characteristics of Installations for Radiotherapy with Fast Neutrons and Other Types of High-LET Radiation

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THREE new types of radiation, neutrons, heavy ions and negative pi-mesons, are at present being studied as alternatives to the conventional radiations in current use for radiotherapy. The conventional radiations, X-rays, gamma rays and electrons, can all be classed as sparsely-ionizing. The new types of radiation either offer a better distribution of dose in the patient (heavy ions and negative pi-mesons) or are densely ionizing (fast neutrons), or both (negative pi-mesons and heavy ions under certain conditions). The term "high-LET radiation" indicates that the density of ionization is high enough to produce a substantial change in biological dose–effect relations, when compared with the conventional radiations. As a rough guide 500 MeV cm^2/g (50 keV/μm) may be taken as the boundary between "low" and "high" values of LET. Of the three new radiations only neutrons are currently in use for radiotherapy.

A total number of 18 abstracts have been submitted for this session, 3 concerning negative pi-mesons and 15 concerning fast neutrons [1]. There are no abstracts about heavy ions or thermal neutron capture therapy. As far as fast neutrons are concerned, radioactive sources and californium are not included in this session as the neutron energy and intensity are inadequate for external beam therapy.

Accepted 22 February 1974.

To make a satisfactory clinical trial of a new quality of radiation it is essential that in respects other than the change in quality under test, the beam should be not significantly worse than conventional beams in current use. Otherwise any possible advantage of the new quality may be hidden by other disadvantages introduced by the new beam. Thus the dose rate, penetration in tissue, and collimation of the new beam must be adequate for effective radiotherapy.

In the case of fast neutrons, the minimum acceptable dose rate can be estimated from clinical experience at Hammersmith Hospital. At 3 fractions per week, 120 rad per treatment are given to the tumour. If the longest practicable treatment time is 10 min, the minimum dose rate at the tumour is 12 rad/min. Most patients are treated with wedge filters; additional filtration may be needed for structural reasons or for beam flattening, and there is attenuation by the overlying tissues. If a factor of approximately 2 for these considerations is allowed, the minimum practical intensity in the unfiltered beam is 25 rad/min.

Collimation and shielding are particularly severe problems with fast neutrons. Even with the most effective shielding material, collimator thicknesses in excess of 50 cm are required. The criteria in this respect are more severe than with sparsely-ionizing radiation because RBE rises as the dose falls. When coupled with

the need for a good depth–dose distribution, this requires a target-to-skin distance of at least 1 metre. For neutron generators employing the $^{3}H(d,n)^{4}He$ reaction, producing neutrons with energies between 14 and 15 MeV, a dose rate of 25 rad/min at 1 metre entails an output of 8×10^{12} n/sec.

Of the 5 discussion abstracts on D–T neutron generators, none of the accelerators projects an output of 8×10^{12} n/sec, the maximum output mentioned being $2{\cdot}5 \times 10^{12}$ by Franke *et al.* The use of a recirculatory gas target, as suggested by Kelsey *et al.*, may enable the required output to be obtained, but the method has not yet been developed to the point where the maximum output can be assessed. The other 3 abstracts in this session (Breynat and Dubus, Broerse *et al.*, and Greene and Jones) describe types of equipment in use now or which can be developed soon, but which are likely to be of value only for biological and physical experiments and a modest programme of experimental radiotherapy.

The fact that there are 10 contributions on the use of cyclotrons, compared to 5 on D–T neutron generators, reflects the inadequacy of the latter technique at the present time. The papers presented at this meeting show that the beam intensity from cyclotrons is adequate for effective therapy but that existing beams lack the flexibility in terms of angulation and continuously adjustable field size that the therapist finds so valuable with conventional equipment. Also neutron beams do not give such good depth doses as ^{60}Co gamma rays unless the deuteron energy is at least 30 MeV.

For low-energy machines the penetration can be improved to some extent by using nuclear reactions different from the usual D on Be reaction. Canada *et al.* have assessed the possibilities of the ^{3}He on Be reaction and found a useful increase in penetration but at the expense of a rather low intensity and high gamma ray contamination. Lorenz *et al.* propose to use a deuterium gas target with 10·5 MeV deuterons, which will be a valuable advance if the method is practicable. Abel *et al.*, Wootton *et al.* and Smathers *et al.*, describe beams generated by deuterons from 13·5 to 50 MeV on beryllium. These beams are in use for therapy at the present time. Attix *et al.*, Jongen *et al.*, Tsunemoto *et al.* and Harrison describe beams generated by deuterons with energies from 35 to 70 MeV which are still being developed. Chaudhri *et al.* present some calculations concerning the potentialities of the $^{7}Li(p,n)^{7}Be$ reaction as a neutron source for radiotherapeutic applications.

There are 3 abstracts on plans for negative pi-mesons; no beams are available at present with the necessary intensity. Kaplan *et al.* describe a visionary idea for generating negative pi-mesons with 500 MeV electrons from a superconducting linear accelerator, together with maximum efficiency of use of the emitted mesons and arrangements for irradiating any selected volume in the patient. Skarsgard *et al.* describe the possible use of a meson channel from a high current 500 MeV proton accelerator. Knapp discusses possible sources of negative pi-mesons, generated by protons or electrons with high or low collection efficiency, and concludes that the cost is similar in all cases.

Although no abstracts on heavy ions were submitted we should not forget their possible value, particularly in improving depth–dose distributions. Table 1 gives approximate values of the currents and energies needed to deliver 60 rad/min at 15 cm depth for a 20×20 cm field. This matter is discussed further in a paper by Todd [2].

Table 1. *Characteristics of heavy ion beams, producing a dose rate of* 60 *rad/min at* 15 *cm depth for a* 20×20 *cm field*

	^{1}H	^{4}He	^{14}N	^{20}Ne	^{40}Ar
MeV/amu	150	150	310	380	520
GeV	0·15	0·6	4·3	7·6	21
pA (fully stripped)	400	200	100	80	50
Entry LET MeV-cm²/g	5·5	22	170	3.0	900

REFERENCES

1. Discussion on characteristics of installations for radiotherapy with fast neutrons and other types of high-LET radiation. *Europ. J. Cancer* **10,** 253 (1974).
2. P. Todd, Radiobiology with heavy charged particles directed at radiotherapy. *Europ. J. Cancer* **10,** 207 (1974).

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Characteristics of ^{252}Cf Neutron Sources Used for Radiotherapy

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Abstract—*Medical neutron sources using ^{252}Cf are currently available for both intracavitary and interstitial radiotherapy. Neutron absorbed dose distributions have been measured primarily with applicator tube sources but are little affected by variations in source encapsulation. Agreement among various laboratories is poorer for neutrons than for gamma rays, which typically contribute only about one third of the total absorbed dose near the source. Recommended neutron dosimetry data, corrected for geometrical attenuation, show a slowly varying exponential decrease with distance from the source, with a value at 1 cm of about 2·4 rad cm^2 μg^{-1}hr^{-1}. Measured average LET's range from 30 to 45 keV μ^{-1}, depending on the contribution of gamma rays to the dose. A new, small afterloading cell for interstitial brachytherapy will incorporate onlyen ough beta ray filtration to reduce the beta–gamma dose rate at 0·5 cm to approximately the neutron dose rate level.*

THE HIGH specific activity for decay by spontaneous fission makes ^{252}Cf a prolific neutron source, readily compatible with the source sizes and source strengths required for implant radiotherapy. In order to explore this potential the U.S. Atomic Energy Commission four years ago launched its biomedical program for ^{252}Cf as one part of a comprehensive market evaluation program dealing as well with industrial, government, and educational applications. The biomedical program now includes fourteen institutional participants in the U.S., England and Japan. A major component of the effort to date under this program has concerned the determination of neutron and gamma ray absorbed dose distributions about ^{252}Cf medical neutron sources [1].

^{252}Cf is an alpha emitter with a 2·73 year half-life and a neutron emitter by virtue of its spontaneous fission decay with a half-life of 85 years. The resulting effective half-life is 2·65 years. On the average 3·76 neutrons accompany each fission, with a fission spectrum having a mean energy of 2·35 MeV. The specific emission rate for neutrons, $2{\cdot}31 \times 10^{12}$ n sec^{-1}g^{-1}, exceeds by orders of magnitude the corresponding rate for (α,n) sources which have been proposed for neutron radiotherapy [2]. Gamma rays from ^{252}Cf are roughly 50% prompt fission gamma rays and 50% fission-product gamma rays.

The medical neutron sources currently available through the AEC loan program include applicator tubes of active length 1·5 cm, needles of active length 2·0 cm, and afterloading cells of active lengths 1·5 cm and 3·0 cm. These sources are made by electrodeposition of ^{252}Cf oxide on Pt–Ir, using multiple wires in needles and applicator tubes in order to achieve high emission rates. Applicator tube sources, intended for intracavitary treatments, are available at source strengths up to 20 μg. Needles are available at up to 5 μg and afterloading cells at up to 0·5 μg cm^{-1}. The cells (0·95 mm dia) are intended to be mounted in plastic tubing and afterloaded into larger-diameter plastic tubes which have been surgically implanted in tissue. A smaller diameter afterloading cell of active length 0·4 cm and total length 0·6 cm is under development at Savannah River Laboratory. The new cell will contain a core of ^{252}Cf–palladium cermet wire and will be singly encapsulated in a Pt–Ir sheath just thick

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enough to reduce beta ray penetration to an acceptable level. The new cells will be fixed in position in a plastic "source tube" prior to shipment to the user.

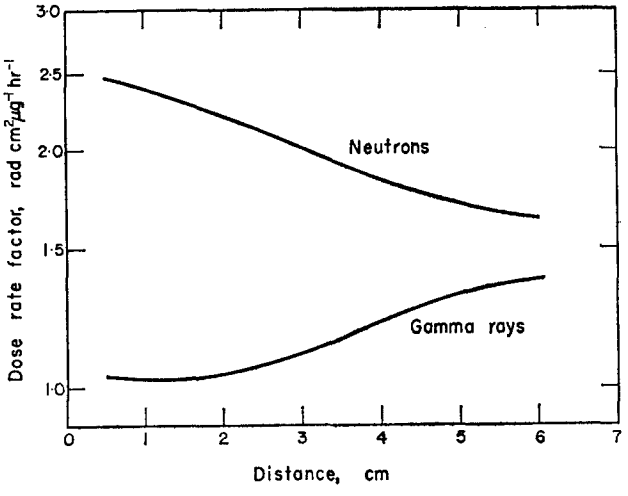


Fig. 1. ^{252}Cf source transverse axis absorbed dose rate, neutron and gamma ray measurements corrected for geometrical attenuation, data from Colvett et al. [3]. *Gamma ray data corrected also to 0·5 mm Pt filtration.*

Recommended values of neutron and gamma ray dose rates for ^{252}Cf medical sources are those plotted in Fig. 1. These data were measured in tissue equivalent fluid by Colvett and co-workers, using parallel-plate ion chambers having cavities 3 mm dia and 0·25 mm thick and using gas multiplication to increase ion currents to measurable levels [3]. Neutron dose rates were derived by difference from a total dose measurement with a chamber of tissue equivalent material and a gamma ray dose measurement with a chamber of aluminum walls and argon gas. The measurements were made using an applicator tube source of 1·5 cm active length. Dividing the transverse-axis data by the geometrical attenuation factor, as in the figure, removes most of the dependence on source length and brings values closer together for easier evaluation. Within the range of distance shown, exponential attenuation is observed to outweigh scattering buildup in the case of neutrons, whereas the geometry-corrected gamma ray dose rate actually increases with distance due to the increasing importance of neutron-capture gamma rays.

In relation to ^{252}Cf dose rate distributions determined at other laboratories, the data of Colvett and colleagues, are on the average, higher for neutrons and lower for gamma rays. Overall agreement among the various measurements and calculations ranges from 10 to 40%, depending on the distance from the source [1]. For neutrons, unfortunately, the spread of values is greatest near the source and, at a distance of 0·5 cm, is about four times larger than the ±5% level of agreement believed essential among institutions using ^{252}Cf in human radiotherapy. For gamma rays the agreement is better near the source (with a spread of 13% at 0·5 cm) and may probably be considered adequate since gamma rays are much less important than neutrons to the biologically effective dose.

Measurements of linear energy transfer (LET) distributions for mixed neutrons and gamma rays from medical ^{252}Cf sources have shown a dose-average LET in the range of 30–45 keV μ^{-1}, the value depending on conditions of measurement [4,5]. The average LET for measurements in tissue equivalent phantom decreases with distance from the source, is lower than for corresponding measurements in air, and is lower for more lightly encapsulated sources. Lower values are in each case correlated with an increased contribution of gamma rays to the total dose.

The new ^{252}Cf afterloading cells using cermet wire will be small enough in diameter for the tube containing them to be inserted easily into implanted tubing of the size currently used for ^{192}Ir implants. Since ^{252}Cf will be compared with ^{192}Ir in clinical trials, it is important to maintain the same dimensional parameters in the implant technique. An earlier version of the small afterloading cell, singly encapsulated in stainless steel of 0·5 mm outside diameter, proved to have unacceptably high beta ray emission from the equilibrium mixture of ^{252}Cf fission products. Measurements using LiF thermoluminescent dosimeters show a total beta-plus-gamma ray dose rate of 44·5 rad $\mu g^{-1}hr^{-1}$ at 0·5 cm from the source along the transverse axis. The geometry-corrected value is 11·7 rad $cm^2\mu g^{-1}hr^{-1}$, a factor of ten higher than the dose rate from gamma rays alone (see Fig. 1). This value is reduced to 2·8 rad $cm^2\mu g^{-1}hr^{-1}$ by a filter of 0·23 mm Pt, which is the filtration selected for the new source final design.

Thus, ^{252}Cf medical neutron sources are available for a wide range of brachytherapy applications. Excluding geometrical attenuation, the neutron dose from such sources decreases slowly with distance from the source source. The n/γ dose ratio is greater than 2·0 near the source but decreases to 1·2 at 6 cm. Small interstitial sources soon to be available will have roughly equal neutron and beta–gamma doses near the source.

REFERENCES

1. L. L. ANDERSON, Status of dosimetry for ^{252}Cf medical neutron sources. *Phys. in Med. Biol.* **18,** 779 (1973).
2. K. H. ANSELL and E. G. HALL, Recent developments in (α,n) sources. In *Neutron Sources and Applications*, Vol. II, p. I–90, CONF-710402, Savannah River Laboratory, Aiken, South Carolina (1973).
3. R. D. COLVETT, H. H. ROSSI and V. KRISHNASWAMY, Dose distribution around a californium-252 needle. *Phys. in Med. Biol.* **17,** 356 (1972).
4. G. D. OLIVER, JR., W. M. QUAM and W. O. WILDE, Empirical dose quality distributions of californium-252. *Hlth. Phys.* **22,** 341 (1972).
5. J. F. DICELLO, W. GROSS and U. KRALJEVIC, Radiation quality of californium-252. *Phys. in Med. Biol.* **17,** 345 (1972).

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Radiobiology with Heavy Charged Particles Directed at Radiotherapy

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Abstract—*Work is beginning in the United States directed at the treatment of tumours other than those of pituitary and brain using range-modulated heavy charged particles. The biological effectiveness and the oxygen dependence of the mixed radiations in the treatment zone are being evaluated by studying the inactivation of human cells in three-dimensional cultures. Experimental depth–survival profiles are presented for range-modulated protons, helium ions, and oxygen ions. In the cases of oxygen and helium ions a decreased O_2 dependence and increased effectiveness are observed. The use of a flat dose profile in the treatment zone does not necessarily lead to a flat survival profile.*

INTRODUCTION

ALTHOUGH protons and helium ions have been used for a number of years in specialized applications such as treatment of tumours of pituitary and cervix, these particle beams have not yet been extensively exploited for general application in radiotherapy [1–3]. Beams of protons and helium ions are therefore currently undergoing biological re-evaluation, in consideration of their possible more widespread application in tumour therapy. In addition beams of heavier ions are undergoing preliminary evaluation for their possible application to tumour treatment. It is the purpose of this presentation to briefly describe charged particle beams available for radiotherapy in the United States and their evaluation particularly with respect to cell inactivation.

Protons

High-energy proton beams of the Uppsala and Harvard synchrocyclotrons have been tested over a number of years in the treatment of tumours of cervix, pituitary, and brain [1,2]. The Harvard Cyclotron has been for several years a full time biomedical research facility, and it has been applied to the treatment of pituitary tumours with Bragg-peak protons. A very small increase in biological effectiveness and a very small decrease in OER is expected in proton irradiations. Proton beams therefore offer mainly superior dose localization. It is reasonable to suspect that superior dose localization, particularly with an absence of exit dose, might decrease the complication rate in treatments of lesions in the vicinity of critical structures such as spinal cord and bowel. Before diversifying the use of proton beams, however, several investigations are necessary. First, the enlargement of the beam for the treatment of areas considerably larger than the typical pituitary tumour is necessary. Ridge filtering (or wedge modulating) to equalize the dose distribution is also necessary. Beams modulated in range need to be evaluated biologically prior to direct application to patients. Studies of the spatial distribution of biological effect in appropriately modulated beams are therefore underway using 3 dimensional cultures of human cells. The dose distribution and cell survival distribution of a modulated proton beam at the Harvard Cyclotron are shown in Fig. 1(a).

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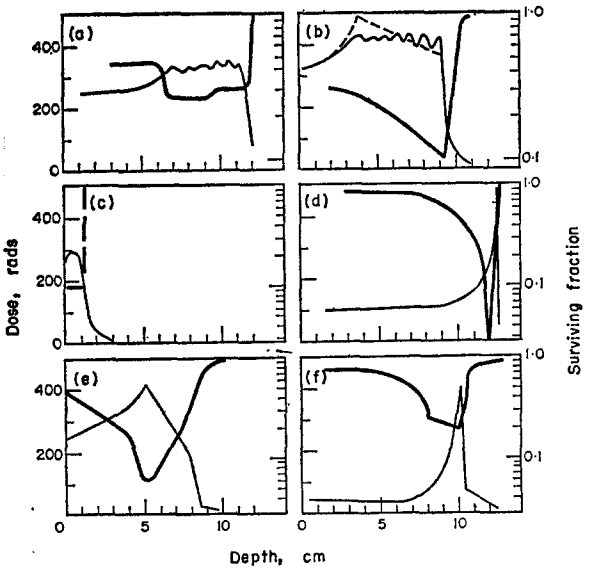


Fig. 1. Experimental and therapeutic depth–dose profiles of various charged-particle beams. Each dose profile (light curve) is accompanied by its resulting cell survival profile (heavy curve). (a). Protons of the Harvard University Synchrocyclotron, (b). Helium ions of the Berkeley Synchrocyclotron [5], *(c). Helium ions of the Space Radiation Effects Laboratory Cyclotron* [6], *(d). Nitrogen ions of the Princeton Particle Accelerator (synchrotron, no range modulation* [12], *(e). Oxygen ions of the Berkeley Bevatron (synchrotron, arbitrary dose scale, hypoxic cells* [14]), *(f). Argon ions, theory and calculation only, no range modulation (data from* [16]).

Helium ions

A considerable amount of experience in irradiating patients with high-energy (910 MeV) helium ions has already been gained at the Berkeley Synchrocyclotron in a program dedicated to the treatment of disorders involving the pituitary, including pituitary tumours [3]. Only recently has this beam undergone trial in treatment of tumours of other tissues [4]. Like protons, helium ions offer a more favourable distribution of deposited energy than conventional radiations. In addition, helium ions appear to be slightly more effective in the killing of cells and slightly less dependent on the presence of oxygen than protons or conventional radiations. For therapeutic applications, as with protons, it is necessary to modulate the helium ion Bragg peak in order to encompass a sufficient treatment volume with the high dose portion of the helium ion's depth–dose profile. When this is done a considerable dilution of the increased biological effectiveness and decreased oxygen dependence of the Bragg peak region of the beam occurs. The extent to which this occurs has been tested biologically using human cells in culture [5]. As in the case of Fig. 1(a) for protons, Fig. 1(b) describes experiments in which the biological effect (human cell survival) as a function of position in a modulated helium ion Bragg peak was determined. Figure 1(b) presents a depth–dose profile and its corresponding depth–survival profile for a helium Bragg peak transformed to give a treatment zone that is 5·5 cm thick. Because of the increased biological effectiveness of Bragg peak helium ions it can be seen that cell survival is higher in the proximal region of the transformed peak than in the distal regions. Ridge filters or rotating wedges designed for Bragg peak modulation of helium or heavy ions for therapy will therefore need to be designed in such a way that the dose delivered in the proximal region of the transformed Bragg peak is higher than the dose delivered at the distal portion. For example, the dashed line in Fig. 1(b) shows the depth–dose profile that would be required to give constant cell survival throughout the modulated Bragg peak. An alternative approach to obtain an equal biological effect in the treatment zone would be to use parallel opposed portals. This latter approach has the advantage of equalizing the biological effectiveness and OER throughout the treatment zone.

In addition to the Berkeley program which is still dedicated primarily to pituitary irradiations, a more systematic series of patient trials is under way at the Space Radiation Effects Laboratory (SREL) cyclotron using helium ions of 700 MeV degraded by absorbers and a ridge filter to give the depth–dose profile shown in Fig. 1(c). The penetration of the beam is shallow because the beam is being used to treat exclusively a skin malignancy, mycosis fungoides. The depth–dose profile of Fig. 1(c) is designed to simulate the depth–dose profile of electrons normally used in this treatment [6]. The trial is presently being executed in the following manner. Each patient that is a candidate for trial has a diseased portion of skin assigned to treatment with electrons and another diseased portion assigned to treatment with helium ions so that each patient is his own control. The same dose and fractionation schedule is used in both zones except that an appropriate RBE (1·4) is applied to the helium ion doses. The response of normal and malignant skin is evaluated by examination as frequently as possible during and after the treatment series, and the treatment is adjusted as necessary to produce a favourable response. It is presumed in this study that, since the depth–dose profiles of the electron and helium ions are identical and since the increased effectiveness of the helium ions (a factor of 1·4) is taken into account, the only factor that is

being evaluated is the role of hypoxia in the survival of malignant cells. This is assumed on the basis of the reduced OER found in previous work [5].

Possibilities for large scale therapeutic trials of helium ions in the U.S. are limited, due to the uncertain future of the SREL accelerator and due to the commitment of the Berkeley program mainly to the treatment of pituitary conditions and the diversion of effort toward heavier ions. Progress therefore in the next few years will be made in small increments.

Nitrogen ions

In 1971 the Princeton Particle Accelerator and the University of California Bevatron successfully accelerated nitrogen ions to energies in excess of 3 GeV [7, 8]. Dosimetric and biological experiments were done and were extensively reported in the literature. The depth–dose profile and depth–survival profile, shown in Fig. 1(d), confirmed that there is an extremely high Bragg peak in the particle stopping region [9–11] and that this high dose region is also more effective in killing human cells [12]. The work of Hall *et al.* [13] showed that there is also a reduced dependence upon oxygen in the high dose region of the beam and that the absolute value of the OER for Bragg peak nitrogen ions probably lies between 1·1 and 1·3. Modulated nitrogen-ion Bragg peaks have not been studied biologically. If the ideal treatment beam is to possess an optimum combination of spatial distribution of dose and oxygen dependence then it is likely that nitrogen ions are superior to helium ions but heavier ions may be better yet.

Oxygen ions

Oxygen ions are being accelerated to GeV energies in the Bevatron at Berkeley 2 or 3 times per year. In addition to a variety of biological and dosimetric studies with mono-energetic oxygen ions, preliminary cell inactivation studies have been carried out using a ridge-filtered oxygen ion beam [14]. Human kidney cells were irradiated at various depths in the presence and absence of oxygen. The depth–dose profile that was achieved and the resulting depth–survival profiles are shown in Fig. 1e. This study suggests also that the OER is significantly lower than that of conventional radiation due to the presence of Bragg peak heavy ions, even when mixed with plateau ionizing heavy ions which have nearly the same OER as conventional radiations.

Argon ions

Earlier, very rough calculational studies indicated that the optimum charged particle for radiotherapy might be neon, due to an apparent favourable combination of spatial dose distribution and low OER [15]. Similar calculational evaluation of argon ions was not carried out, due to the decreasing RBE of argon ions as a function of depth, and due to the attenuation of argon ion beams by nuclear interactions. Nevertheless, argon ions have OER values of about 1·0 throughout significant lengths of their tracks. It is anticipated that argon ions will provide a radiation modality with a spatial profile slightly superior to that of gamma rays and a minimum oxygen dependence. By using existing data on cell inactivation with low energy heavy ions a depth–survival profile for a 50 rad surface dose of argon ions can be derived as shown in Fig. 1(f). In the particle stopping region there is a moderately intensified killing of cells. The same curve also applies to the survival of hypoxic cells in the last 2 cm of the beam path. The OER is greater than 1·0 at smaller depths.

THE PROSPECTS FOR HEAVY ION RADIOTHERAPY

The number of accelerators in the world with known capability of accelerating a specific type of ions is approximately inversely proportional to a high power of the ion's mass. For ions heavier than helium adequate penetration requires energies in the GeV range. World-wide availability of ions heavier than helium therefore currently depends on the availability of large synchrotrons. In case future applications are indicated, advanced technological concepts such as cryogenic accelerators or electron ring accelerators will need to be developed. Cryogenic and plasma accelerators will presumably have the advantage of being small and therefore adaptable to installation in a heavily shielded hospital basement. However, neither the feasibility nor the cost of such devices is presently known.

Many accelerators are experiencing diminished utilization in the area of intermediate energy physics research, due to the reduced interest in this area, the reduced support of physics research in general, and the development of modern high intensity intermediate energy accelerators. Total operating costs for these accelerators is in the vicinity of 100–200 dollars per hr. Some of the accelerators are in

or near urban centres but many are in outlying locations and would be useful, if at all, only in treating ambulatory patients. Proposals have been made for the construction of inexpensive synchrocyclotrons specialized for use in hospitals. In addition to possible utilization for tumour therapy these machines would also be capable of producing short lived proton-rich radioisotopes and of being used for surgical procedures involving the pituitary.

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REFERENCES

1. S. Graffman and B. Jung, Clinical trials in radiotherapy and the merits of high energy protons. *Acta Radiol., Ther. Phys. Biol.* **9,** 1 (1970).
2. A. M. Koehler and W. M. Preston, Protons in radiation therapy. *Radiology* **104,** 191 (1972).
3. J. A. Linfoot, C. Y. Chong, J. F. Garcia, A. S. Cleveland, G. M. Connell, E. Manougian, M. D. Okerlund, J. L. Born and J. H. Lawrence, Heavy particle therapy for acromegaly, Cushing's disease, Nelson's syndrome, and non-functioning pituitary adenomas. *Prog. atomic Med.: Recent Advan. Nucl. Med.* **3,** 219 (1971).
4. C. A. Tobias, J. T. Lyman and J. H. Lawrence, Some considerations of physical and biological factors in radiotherapy with high-LET radiations including heavy particles, pi mesons, and fast neutrons. *Prog. atomic Med.: Recent Advan. Nucl. Med.* **3,** 167 (1971).
5. M. R. Raju, M. Gnanapurani, B. Martins, J. Howard and J. T. Lyman, Measurement of OER and RBE of a 910 MeV helium ion beam using cultured cells (T-1). *Radiology* **162,** 425 (1972).
6. L. Z. Nisce, G. J. D'Angio and J.-H. Kim, Weekly total skin electron irradiation for mycosis fungoides. *Radiology*, **109,** 683 (1973).
7. M. G. White, M. Isaila, K. Prelec and H. L. Allen, Acceleration of nitrogen ions to 7·4 GeV in the Princeton Particle Accelerator. *Science* **174,** 1121 (1971).
8. H. A. Grunder, W. D. Hartsough, and E. J. Lofgren, Acceleration of heavy ions at the Bevatron. *Science* **174,** 1129 (1971).
9. C. A. Tobias, J. T. Lyman, A. Chatterjee, J. Howard, H. D. Maccabee, M. R. Raju, A. R. Smith, J. M. Sperinde and G. P. Welch, Radiological physics characteristics of the extracted heavy ion beams of the bevatron. *Science* **174,** 1131 (1971).
10. R. C. Rogers, J. F. Dicello and W. Gross, The biophysical properties of 3·9 GeV nitrogen ions—II. Microdosimetry. *Radiat. Res.* **54,** 12 (1973).
11. W. Schimmerling, K. G. Vosburgh and P. Todd, Interaction of 3·9-GeV nitrogen ions with matter. *Science* **174,** 1123 (1971).
12. D. Q. Brown, H. G. Seydel and P. Todd, Inactivation of cultured human cells and control of C3H mouse mammary tumors with accelerated nitrogen ions. *Cancer,* **32,** 541 (1973).
13. E. J. Hall, W. Gross, H. H. Rossi and A. M. Kellerer, OER and RBE determinations with a 3·9 GeV nitrogen ion beam. *Radiat. Res.* **51,** 450 (1972).
14. R. Roisman, B. I. Martins, D. Kalafonos and C. A. Tobias, Cell survival as a function of depth for a 220-Mev/nucleon oxygen-ion beam. *Radiology*, (In press).
15. C. A. Tobias and P. W. Todd, Heavy charged particles in cancer therapy. *Natl. Cancer Inst. (U.S.A.) Monogr.* **24,** 1 (1967).
16. P. Todd, Heavy ion irradiation of cultured human cells. *Radiat. Res. Suppl.* **7,** 196 (1967).

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Negative Pions in Radiotherapy: A Brief Review

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Abstract—*The frequency and energy distribution of fast-neutron interaction products such as protons, alpha particles, and heavy recoils and those arising from negative pion absorption processes are similar. Because of these similarities in radiation quality of negative pions and fast neutrons in the treatment volume, the therapeutic experience of fast neutrons is relevant for negative pions as well. However, in the case of negative pions, dose deposited in the treatment volume extends to much lower LET values than for fast neutrons because a significant fraction of the dose is deposited by energy losses of negative pions. The biological effectiveness for nearly monoenergetic negative pions at the peak of the depth–dose distribution was found to be significantly higher than at the entrance. For pion beams of larger momentum spread that are therapeutically relevant, the differences in biological effectiveness are reduced considerably; however, a significant reduction in OER still can be expected. Considerable changes in biological effectiveness within the peak region have been found. These changes will be reduced when opposed fields of negative pions are used. Although the current knowledge on biological effects sheds some light regarding what to expect when negative pion beams are used in therapy, more work needs to be done with intense negative pion beams before its possible therapeutic use.*

INTRODUCTION

THE PURPOSE of this presentation is to review briefly the physical and biological aspects of negative pions and to describe the similarities of fast neutrons and negative pions in their radiation quality in the treatment volume when used in radiotherapy. For possible improvements in radiotherapy, we are looking for various techniques that will minimize the unavoidable dose to surrounding normal tissues and, in addition, we would like to produce more effect on tumour cells for a given effect on normal tissues within the treatment volume. In principle, negative pions offer both these possibilities.

Negative pions with energies of 60–90 MeV (range 12–23 cm in tissue) are of interest in radiotherapy and deposit their dose mainly by energy loss phenomena in the 0·3 keV/μ LET region, although a small but significant fraction of the dose is deposited at higher LET due to nuclear interactions. As they pass through a medium such as tissue, negative pions slow down, and the dose deposited by them increases slowly and reaches a maximum near the end of the range known as the Bragg peak. The LET due to energy losses of negative pions increases to about 5 keV/μ. Near the end of the range, negative pions are absorbed by the nuclei of the medium, and their rest mass of 140 MeV appears in the form of kinetic energy of nuclear fragments except for about 40 MeV, which is used in overcoming the binding energy of the nucleus. Nearly 60 MeV appears in the form of kinetic energy of neutrons, and the remaining 40 MeV appears in the form of of kinetic energy of charged particles such as protons, deuterons, tritons, alpha particles, and heavy nuclei that are absorbed locally. This unique property of negative pions makes them promising in radiotherapy. This was recognized by Fermi and a few others, and detailed calculations by Fowler and Perkins [1] motivated many groups in different parts of the world to measure the physical and radiobiological pro-

perties of negative pions, although the intensity of existing negative pion beams is 100–1000 times less than the intensity required for radiotherapeutic applications. Current interest in the use of negative pions stems from the impending completion of accelerators producing intense sources of this radiation and associated biomedical facilities at Los Alamos, Stanford, Vancouver, and at Zurich, Switzerland. In addition, there are plans to increase the dose rate to about 10 rad/min over a field size of 8×8 cm at the NIMROD accelerator beam in Harwell, England, during 1974.

NEGATIVE-PION ABSORPTION AND NEUTRON INTERACTION PRODUCTS

The type and energy of the particles produced by 14 MeV neutrons and negative pion capture in tissue are shown in Table 1. It can be noted that a number of interaction products are similar, however, a comparison of the radiation quality of fast neutrons and negative pions has to be based on the actual LET spectra of these two radiation modalities.

*Table 1. Charged particle production by 14 MeV neutron interactions and negative pion capture in tissue**

Type of particle	Neutron interaction: Particles produced per interaction (elastic and inelastic)	Neutron interaction: Average energy (MeV)	Negative pion capture: Particles produced per negative pion capture	Negative pion capture: Average energy (MeV)
Protons	0·58	7	1·2	20
Alpha particles	0·10	4	1·2	11
Heavy recoils	0·50	2	0·5	3·2

* T. W. Armstrong, Oak Ridge National Laboratory (private communication, 1973). Results for negative pion capture are from the computer code using the intranuclear-cascade-evaporation model [2] and for 14 MeV neutrons from Caswell and Coyne [3]. In case of negative pions, there are also a total number of 0·36 light ions such as deuterons, tritons, and ^{3}He produced per capture.

EXPERIMENTAL WORK

Most of the past experimental work on therapeutic aspects of negative pions was done at Berkeley, and at CERN, Switzerland, using negative pion beams designed for physics experiments. These beams were nearly monoenergetic (~5% momentum spread). The depth–dose distribution of such beams gives insufficiently wide Bragg peaks to cover most of the treatment volumes in radiotherapy. The program at Berkeley subsequently was transferred to Los Alamos. The program at CERN is directed especially at problems relevant to radiation protection. A special negative pion beam facility for biomedical work was recently built at the 7 GeV proton synchrotron (NIMROD) in England. This beam has a momentum spread of about 13%, with a wider Bragg peak than at Berkeley, and a dose rate of about 60 rad/hr. Lindop, Berry, Nias, and associates, in collaboration with the Health Physics Group of the Atomic Energy Research Establishment at Harwell, England, have recently performed a number of experiments on the radiobiology of negative pions. Oak Ridge National Laboratory is using Monte Carlo techniques to compute depth–dose, LET distributions, and cell survival for negative pion beams of interest in therapy.

Dosimetry

The depth–dose distribution measurements at Berkeley and at CERN gave peak-to-plateau ratios in the region of 2–3 with a peak width of about 2 cm. With increasing momentum spread of the negative pion beam, the peak width increases and the peak-to-plateau dose ratio decreases. Experimental depth–dose distributions agree well with theoretical calculations [2–4]. Figure 1(a) shows the calculated depth–dose distribution for a pion beam of a momentum spread chosen such that the dose is nearly uniform in the depth interval from 12·5 to 17·5 cm [5]. Figure 1(b) shows the per cent of the dose deposited by events with a LET greater than the threshold LET. A plot for 14 MeV neutrons and for ^{60}Co gamma rays also is shown in the figure for comparison. Most of the dose at the plateau, except for a small fraction, is nearly of the same LET as

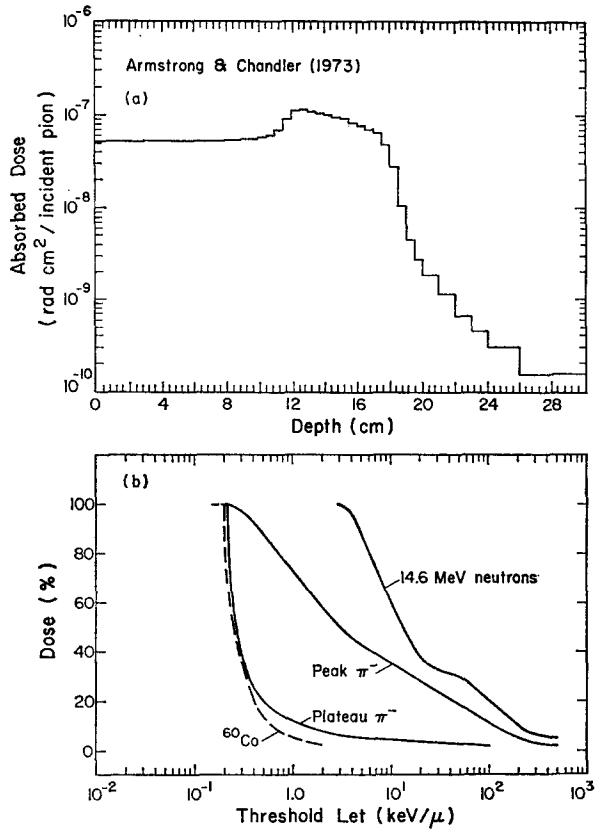


Fig. 1. *(a) Calculated depth–dose distribution of negative pions; and (b) integral LET spectra for negative pions at plateau and peak, fast neutrons, and* ^{60}*Co gamma rays. Such a plot gives the per cent dose contribution due to events greater than the threshold LET.*

that of ^{60}Co gamma rays, whereas a significant fraction of the dose at the peak is characterized by LET values in excess of 10 keV/μ, similar to that of fast neutrons. In case of fast-neutron interactions, all the dose deposited is due to interaction products, whereas for negative pions a significant fraction of the dose is deposited by energy loss of negative pions before capture. Hence, the LET in the pion capture region extends to much lower LET than for fast neutrons.

Radiobiology

Most of the radiobiological measurements of negative pions at Berkeley and CERN were done at the peak of the depth–dose distribution. The beams had a momentum spread of about 5%, and the dose due to contamination (μ^- and e^-) was about 15% of the total dose at the peak. The dose rates at Berkeley and CERN were about 60 rad/hr and 3 rad/hr, respectively [6]. The beam used in England had a wider momentum spread (13%), but beams of even wider spread will be necessary for applications in therapy. The RBE and OER data for pions are shown in Table 2. Depending on the biological end point and the system used, the RBE value at the peak was in the region of 1·4–5·0. The

Table 2. *Summary of RBE and OER values of negative pions at the peak of depth–dose distribution obtained by various authors for different biological systems*

Biological system	RBE	OER
BERKELEY		
Abnormal anaphases in *Vicia faba* root meristems	2·4	
Chromatid aberrations in *Vicia faba*	3·0	1·8
Vicia faba 10-day growth	3·0	1·35–1·5
Arginine reversions in yeast	1·4	1·9
Proliferative capacity of ascites tumour cells	3·0–5·0	
Polyploidy induction in ascites tumour cells	2·5	
Human kidney cells (frozen)	1·6	
Human kidney cells (room temperature)	2·4	1·6
Human lymphocytes	2·0	
CERN		
Spermatogonia	3·7	
Vicia faba 10-day growth	2·5–4·5	
ENGLAND		
Vicia faba 10-day growth	2·5	1·7–1·8
HeLa cells (frozen)	1·86	
HeLa cells (room temperature)	2·5	

OER values at the peak of the depth–dose distribution were in the region of 1·5–1·9.

Significant differences in biological effectiveness were detected around the peak region by all investigators. Figure 2 shows the results from Berkeley using human kidney cells in culture, CERN results using bean roots, and results from England using bean roots and HeLa cells in culture. The biological effectiveness at the back side of the peak (position III) is

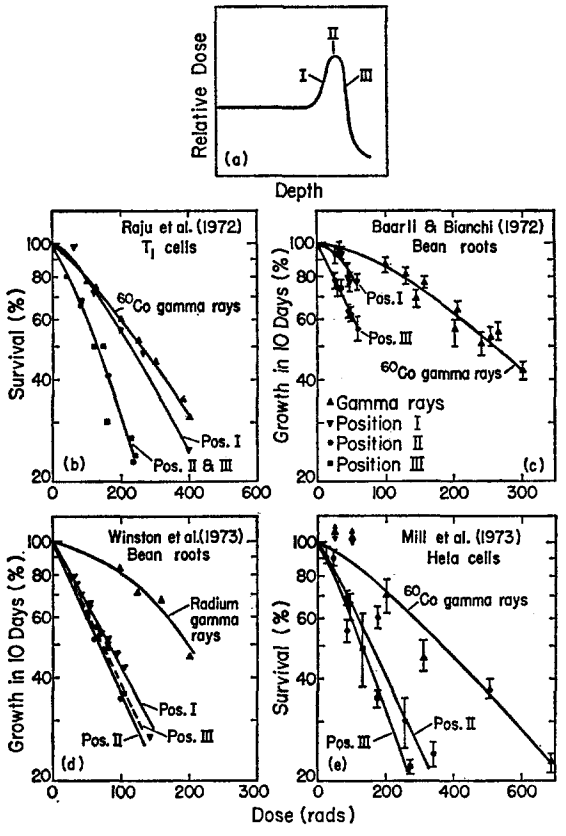


Fig. 2. (a) Schematic depth–dose distribution showing various positions in the peak region where biological materials were exposed; (b) survival curves with T_1 cells measured at Berkeley [10]*; (c) 10-day growth of bean roots measured at CERN* [11]*; (d) 10-day growth of bean roots measured at NIMROD* [9]*; and (e) survival curves with HeLa cells measured at NIMROD* [12].

higher than at the front side (position I). This can be expected because most of the dose in position I is deposited at low LET by energy loss of negative pions, whereas in position III most of the dose is deposited at high LET by negative pion absorption processes. These changes in biological effectiveness have to be taken into consideration in the use of negative pions in therapy, although they will be reduced when two opposed fields are used. Lindop and associates have also done some fractionation experiments at the plateau and peak regions (marked positions II and III in Fig. 2a). Their results indicate that there was practically no recovery at the back of the peak (position III) but that there was significant recovery at the peak (position II) and at the plateau.

The biological effectiveness at the plateau was found to be the same as that for ^{60}Co gamma rays for polyploidy induction in lymphoma ascites cells [7]. Nias *et al.* [8] also found that the RBE at the plateau is nearly 1·0 for frozen HeLa cells. However, Winston *et al.* [9] have reported a significant increase in RBE and decrease in OER even at the plateau region. These differences in biological effects at the plateau could be partly due to differences among the biological systems and end points used in these investigations. More work needs to be done in these areas.

With increasing beam momentum spread, the LET differences between the plateau and peak are diminished. This is due to the increase in dose fraction from energy-loss events in the peak region with increasing momentum spread. In some of the preliminary work of Lindop and associates in England (private communication, 1973), significant differences in RBE between the plateau and peak regions could not be detected when biological criteria such as thymic weight loss, spleen weight loss, and colony-forming units (bone-marrow cells) were applied.

Data from experiments with other beams of radiation that have a wide LET spread indicate that even a small fraction of the high-LET component ($\gtrsim$100 keV/μ) is very effective in reducing the OER significantly, although the RBE does not increase accordingly [10]. Our recent results for various mixtures of X-rays and alpha particles (residual energy ~3 MeV) from plutonium also confirm this finding. These results indicate that, although RBE corrections for therepeutically useful negative pion beams may not be large, a significant reduction in OER is expected.

Acknowledgements—It is indeed a pleasure to thank Professor P. J. Lindop, Dr. R. J. Berry, Dr. D. H. Reading, and Dr. A. H. W. Nias for giving me permission to quote their unpublished results and for their comments to improve this manuscript. I also thank Dr. T. W. Armstrong and D . R. S. Caswell for providing information on fast-neutron and negative-pion calculations. This work was performed under the auspices of the U.S. Atomic Energy Commission and the National Cancer Institute.

REFERENCES

1. P. H. Fowler and D. H. Perkins, The possibility of therapeutic applications of beams of negative π-mesons. *Nature (Lond.)* **189,** 524 (1961).
2. T. W. Armstrong and K. C. Chandler, Monte Carlo calculations of the dose induced by charged pions and comparison with experiment. *Radiat. Res.* **52,** 247 (1972).
3. R. S. Caswell and J. J. Coyne, Interaction of neutrons and secondary charged particles with tissue: secondary particle spectra. *Radiat. Res.* **52,** 448 (1972).
4. J. E. Turner, J. Dutrannois, H. A. Wright, R. N. Hamm, J. Baarli, A. H. Sullivan and S. M. Seltzer, The computation of pion depth–dose curves in water and comparison with experiment. *Radiat. Res.* **52,** 229 (1972).
5. T. W. Armstrong and K. C. Chandler. *Oak Ridge National Laboratory Report* ORNL-TM-4294 (1973) (To be published).
6. M. R. Raju and C. Richman, Negative pion radiotherapy: physical and radiobiology aspects. *Current Topics in Radiat. Res. Quart.* **8,** 159 (1972).
7. W. D. Loughman, J. M. Feola, M. R. Raju and H. S. Winchell, RBE of beams in the Bragg peak region determined with polyploidy induction in mammalian cells irradiated *in vivo*. *Radiat. Res.* **34,** 56 (1968).
8. A. H. W. Nias, D. Greene, D. Major, D. R. Perry and D. H. Reading, Determination of RBE values for fast neutrons and negative π mesons using frozen HeLa cells. *Brit. J. Radiol.* (1973) (Submitted).
9. B. M. Winston, R. J. Berry and D. R. Perry, Response of *Vicia faba* to irradiation with a beam of negative π mesons, under aerobic and hypoxic conditions. *Brit. J. Radiol.* **46,** 541 (1973).
10. M. R. Raju, G. Gnanapurani, B. Martins, J. Howard and J. T. Lyman, Measurement of OER and RBE of a 910 MeV helium ion beam, using cultured cells (T–1). *Radiology* **102,** 425 (1972).
11. J. Baarli and M. Bianchi, Observed variations of r.b.e. values in the stopping region of a 95 MeV negative pion beam. *Int. J. Radiat. Biol.* **22,** 183 (1972).
12. A. J. Mill, P. J. Lindop and J. D. Lewis, (Private Communication) (1973).

Europ. J. Cancer Vol. 10, pp. 217–224. Pergamon Press 1974. Printed in Great Britain

Dose-limiting Tissues in Relation to Types and Location of Tumours: Implications for Efforts to Improve Radiation Dose Distributions

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Abstract—*We consider the potential gains to be expected from improved dose distributions which can, for example, be obtained from large field, low LET, energy modulated proton beams. Increases in tumour control probability which have resulted from improvements in dose distribution in the past are discussed. Limitations on the maximum dose which can be delivered and on possible ways in which normal tissue tolerance can be extended are considered. The impact of improved dose distributions on management of a number of diseases, particularly cancer of the urinary bladder, is discussed. It is concluded that, for sites in which conventional radiotherapy can deliver doses of the order of* 7000 *rad in* 7 *weeks, improvements in local control are unlikely and any improvements in results may be in the form of a reduction of morbidity. However, significantly higher frequency of local control may be achieved in those normal tissue–tumour situations in which doses are currently limited to about* 5000 *rad in* 5 *weeks but could be increased to levels* >6000 *rad in* 6 *weeks by use of improved dose distribution patterns obtainable with proton beams.*

INTRODUCTION

THE CURRENT interest in utilization of heavy ion or negative pion particle beams in radiation therapy rests on the expectation that the reduced oxygen enhancement ratio, lesser shoulder width of cell survival curve, and the superior dose distribution patterns achievable will permit the radiation therapist to obtain better results. An advance will be obtained in the form of either a higher tumour control probability or a lesser probability of significant damage to normal tissue; optimistically, improvement could be better in both categories. If superior results were achieved, a serious question would be: what is the importance of the dose distribution patterns as distinct from the lower oxygen enhancement ratio or smaller shoulder? This point will be considered herewith respect to radiation therapy based on low LET, large field proton beams. Protons are now being studied (in the Soviet Union and Sweden and in the near future in the United States), as a low LET conventionally fractionated radiation therapy technique [1]. Proton radiation therapy is pertinent to this discussion because the dose distributions achievable with appropriately modulated proton beams are roughly comparable to those which could be expected from negative pions and heavy ion particle beams.

This discussion is facilitated because of the RBE for low LET modulated proton beam therapy is 1·0. This statement is based on

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recent work conducted on the Harvard Cyclotron by Dr. Jerry Williams and Mr. Jim Robertson of the Harvard School of Public Health and Dr. Paul Todd of Pennsylvania State University on the same machine. They found the RBE to be 1·0 at various points in depth along the central axis and at the edge of a modulated 160 MeV proton beam. This RBE value of 1·0 was obtained for both low (100–300 rad) and high dose levels. The modulated beam, which was prepared by Mr. Andy Koehler of the Harvard Cyclotron Laboratory, has depth–dose characteristics, indicated in Fig. 1(a), which would be typical of a clinically useful beam. It is characterized by an entrance dose of 87%; the dose rises to 100% at about 5 cm and remains at this level to about 13 cm and then decreases within a few millimetres to less than 5%. Obviously, by the utilization of two or more beams of such characteristics, highly attractive depth–dose distribution patterns could be achieved for irradiation of water-equivalent phantoms.

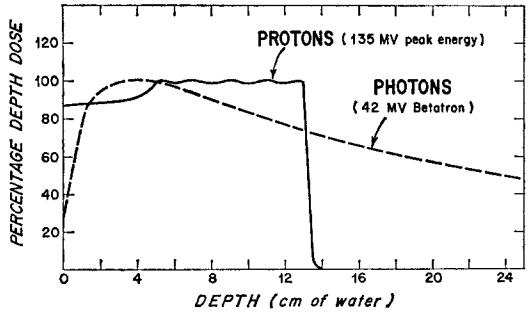


Fig. 1. *(a) Central axis depth dose of a typical energy modulated proton beam. A similar curve characteristic of a 42 MV betatron is shown for comparison.*

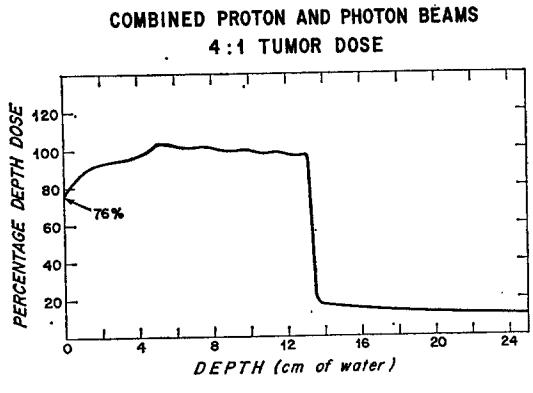


Fig. 1. *(b) Central axis depth dose of combined proton and photon beams.* 20% *of the "tumour dose" is delivered by the 42 MV photon beam.*

In many instances, it would be desirable to irradiate tumours through a single portal. However, if the target volume is to be taken to tumouricidal doses, the high entrance dose of such proton beams would be a disadvantageous and, even, a limiting factor. We have proposed that a combination of proton and high energy (say 42MV) photon beams can overcome this problem. The depth–dose characteristic of one such combination is presented in Fig. 1(b). Here, 20% of the tumour dose is contributed by the photon beam. The combined entrance dose is 76% and the reduction from the 87% entrance dose of the proton beam alone is due to the extremely low photon beam entrance dose which is of the order of 30%. The entrance dose of the combined beams may be lowered further at the expense of increasing the "tail" at large depths.

The basic question being considered here is: would, as a consequence of application of particle beam techniques, the radiation therapist be able to administer a higher dose to the tumour-bearing part because sufficient volume of normal tissue or certain critical normal tissue were excluded from the high dose volume? If so, one would expect that a higher tumour control probability would be obtained since the working hypothesis is that for human tumours, as has been well shown for tumours of experimental animals, there is an orderly and regular increase in tumour control probability with radiation dose, Even if a higher radiation dose could not be given to the tumour-bearing site, the results could be better because the morbidity associated with the treatment might be reduced as a consequence of diminution in the volume of normal tissue irradiated. The point at issue is the degree to which tumour doses are held back from the maximum dose which could be tolerated by the tumour site because of direct or secondary effects of the inclusion of normal tissue in the treatment volume.

HISTORICAL EVALUATION OF THE IMPACT OF IMPROVED DOSE DISTRIBUTIONS ON RESULTS OF RADIATION THERAPY

Carcinoma of the skin

Results of treatment of carcinoma of the skin are virtually unchanged from those which were reported in the 1930's. For example, Mosekilde reported that 384 of 420 lesions (91%) healed primarily following low kV X-ray therapy [2]. Similarly, Ebbehoj reported 168 out of 174 (96%) of skin lesions were controlled locally by use of low kV X-rays [3]. Magnusson, in 1935, described results of treatment of 284 lesions less than 2 × 2 cm by

radium needle implantation [4]. He claimed a 97% corrected three-year survival in this group. Von Essen reviewed the results of the Swedish Tumour Institute in treatment of skin cancer over the period from 1938 to 1955 and found a local recurrence rate of 5% in 494 lesions treated by 120 to 140 kV X-rays [5]. The enormous improvement in technology for radiation therapy over the past 20 years has made no impact on the treatment of carcinoma of the skin. Techniques and results have remained unchanged because dose distribution patterns were excellent with what we would now consider the relatively limited technology available 30–40 years ago.

Carcinoma of the glottis

This is another class of tumour for which treatment results have improved relatively little over the last 25 years. Quite good results were obtained in several clinics with 180–250 kV X-ray technique for early (T1) lesions of the glottis. For example, Nielsen from Copenhagen treated 49 patients with early stage carcinoma during the period of 1931 to 1944. Of these 49, 42 patients were alive at more than 5 years. There was only one local recurrence in this group [6]. As cobalt-60 and low energy linear accelerator equipment became available, they were employed in treatment of glottic carcinoma, but improvements in results of treatment have been limited to reduction of atrophic changes of the overlying skin. Results from cobalt or linear accelerator treatment are not appreciably better in terms of disease-free survival for stage T1 lesions of the glottis than was the case in the 180–250 kV era.

Carcinoma of the urinary bladder

Prior to the advent of supervoltage radiation therapy, there was a negligible role for radiation therapy in the management of patients with carcinoma of the bladder by external beam technique. This is readily understandable since doses to the bladder were restricted to about 4500 rad in 4 weeks. With supervoltage techniques, dose levels commonly employed are in the range of 6000 rad/6 weeks to 7000 rad/7 weeks. Thus, there is no surprise that some significant increases in tumour control and survival results have been achieved by supervoltage radiation therapy alone. Morrison and Deeley of Hammersmith Hospital [7] described results of 8 MeV linear accelerator radiation therapy of T3 and T4 carcinomas of the urinary bladder. T3 lesions being those with perivesicle disease and T4 being those with fixed tumour mass. For T3 and T4 lesions, 21% (of 128 patients) and 19% (of 99 patients) survived four years. Miller and Johnson (M.D. Anderson Hospital) reported 5-year survival figures for radical dose radiation therapy (7000 rad/7 weeks) for primary carcinoma of urinary bladder stage C and D1 to be 26% (of 66 patients) and 10% (of 176 patients) respectively [8]. Accordingly, for patients with bladder carcinoma, supervoltage technology has been a highly important development.

Carcinoma of the uterine cervix

Comparative survival for carcinoma of the uterine cervix treated by radium and orthovoltage or radium and supervoltage over different time periods have been published from the M. D. Anderson Hospital [9]. These are shown in Table 1 and indicate a clinically important advantage for the supervoltage techniques in patients with carcinoma of the uterine cervix stage IIB, IIIA, and IIIB. For early stage disease, radium was the principal therapeutic modality during both periods. As expected, survival figures did not change since technique and dose level were essentially constant.

Table 1. *Results of treatment squamous cell carcinoma of intact uteri*

	Before supervoltage		Supervoltage	
Stage	No. treated	(%) Survival 5 years	No. treated	(%) Survial 5 years
IIB	123	60	114	73
IIIA	93	44	130	56
IIIB	87	31	148	38

After Fletcher [9].

Carcinoma of the prostate

Prior to the supervoltage period, patients with carcinoma of the prostate were rarely treated by radiation therapy. Intensive clinical study of the role of radiation therapy as the primary treatment of localized carcinoma of the prostate is currently being conducted in many clinics. Several reports now document clearance of pelvis of tumour with patients remaining disease-free for at least five years. Such results were not achieved with 250 kV equipment.

Hodgkins' disease

Technique and interest of radiation therapy has changed drastically over the past decade i.e. after use of supervoltage became wide-

spread. To the extent that "total lymph node irradiation" procedures improve survival and that a dose of $\geqslant$4000 rad/4 weeks is necessary, then supervoltage technology represents a major advance. Such a radical approach would not be feasible with orthovoltage equipment because of severity of skin reaction and the total organism damage due to the very large side scatter component of the secondary radiations.

Glioblastoma multi-forme

Five year survival figures remain frighteningly close to zero despite use of supervoltage equipment and dose levels of about 6000 rad in six weeks.

In summary, clinical data for several disease categories demonstrate that when a major increase in radiation dose to the tumour site becomes possible as a result of the use of more advanced equipment, an important increase in tumour response and disease free survival rates have been common. In those situations where there were no or minor changes in the administered dose levels, results have not altered.

POTENTIAL FOR FURTHER IMPROVEMENT BY UTILIZATION OF SUPERIOR DOSE DISTRIBUTION PATTERNS

General considerations

Further increase in tumour dose. If experience of the past 40 years may be taken as prologue, we can only anticipate additional improvements in tumour control probability from employment of better dose distribution patterns to the extent that higher dose levels at the at the tumour site are achieved.

One does not know, at this time, the precise upper limit to which the radiation dose might be carried with safety even when extensive field reductions are employed. However, there are at present few, if any, situations where the radiation dose is regularly carried to more than 7500 rad in seven weeks regardless of field size or treatment volume. In fact, in most situations, a lower dose is considered maximum even for small treatment volumes. From the foregoing, it is evident that local dose cannot be increased without limit and that, for many situations, we are already very close to or at the maximum dose which can be tolerated by the tumour-bearing tissues, viz. 6500–7000 rad in 6–7 weeks as commonly used for lesions of the bladder, prostate, many head and neck sites, etc.

Response of the tumour-bearing structure. The tolerance of the tumour-bearing structure depends not only upon the ability of the cells of the constituent tissues to repair radiation damage *per se* (repair, repopulate, etc.) but also upon the extent of destruction of the tissues by tumour. An organ partially destroyed by tumour growth is less tolerant of radiation than is such an organ that is uninvolved by tumour. An indication of this last mentioned fact is that in many tumour–normal tissue situations, necrosis subsequent to radiation therapy is found to occur at the tumour site and not in some surrounding normal tissue that is being inadvertently irradiated to the same dose level.

The reconstitution of an irradiated tissue is commonly assumed to be affected by whether or not adjacent tissues have suffered irradiation. It is a reasonable hope that a sharp reduction in volume of irradiated nearby normal tissues, or in the proportion of the tumour-bearing structure which is irradiated, could lead to an improved tolerance of the tumour-bearing structure which is subject to irradiation. This, in turn, would permit the application of higher doses to the tumour volume and open the possibility of improved tumour control.

Potential improvements. In summary, we can expect improved therapeutic outcome as a result of employment of improved dose distribution in the following ways:

1. Modestly improved local control from modest increases in dose where the dose is presently high and limited by tolerance of the tumour-bearing structure but can, perhaps, be increased somewhat due to increased tolerance of the tumour site.
2. Major improvements in local control frequency where substantial dose increases are possible compared with conventional photon techniques which are limited because large volumes of low tolerance normal tissues are included in the treatment volume.
3. Reduction in frequency of complications as a consequence of lesser dose to critical surrounding tissues.

For those lesions which are presently treated to so high a dose that (1) and (2) will not apply, improvements in technique will at most be effective in reducing morbidity—this is obviously a wholly desirable and worthwhile gain.

Whether or not the most effective dose distribution can be achieved by special beam techniques, or by combination of high energy photon beams with interstitial techniques is a subject for future study.

DETAILED CONSIDERATION OF CARCINOMA OF THE URINARY BLADDER

As an example, we will now consider in some depth the likelihood of improving results of carcinoma of the urinary bladder if and when idealized depth–dose distribution patterns are available at a clinical level, assuming that effects of tissue heterogeneity will not be a serious problem. The critical question is: what are the sites of complications following radical dose radiation therapy? That is, are they found primarily in the target organ or in structures or tissues which might be irradiated to lesser doses by employing more elegant technology? Table 2 shows five-year survival and local recurrence results amongst the 533 patients with bladder carcinoma treated by radiation therapy alone at the M. D. Anderson Hospital.

Table 2. Five year survival and local failure results in patients with bladder carcinoma treated by radiotherapy alone

Stage	Five year survival	Local failure
B2	15/85 (17%)	45%
C	17/66 (26%)	37%
D1	17/176(10%)	45%

After Miller and Johnson [8].

These results clearly document that a substantial number of patients survive for five years. Importantly, they show that there is a tremendous need for even better results: local failures are common and survival is very much less than 100%. While it is impossible to predict the impact of better dose distribution patterns on local control rate and ultimate survival figures, we do know that prior to supervoltage therapy there were practically no local controls and there were virtually no survivors. It is reasonable to project that if the local failure rate could be reduced substantially as a consequence of a new thechnology, there would be a measurable increase in survival.

The radiation dose employed in these patients has been about 7000 rad in seven weeks in 35 fractions. That significant reactions were produced is clear from the complication frequency and severity as listed in Table 3. The major complication rate was 14% (73/533); for 5% (27) of all patients the complication was fatal. Obviously, dose level can be increased only by different techniques or beam qualities. Note that 22 of these 27 fatal complications were centred in the bladder. There were five patients with complications of bladder alone, eight with both bladder changes and damage in adjacent structures and nine deaths scored as complications secondary to obstructive uropathy, produced by reactions

Table 3. Sites of complications in 533 patients with carcinoma of urinary bladder treated by radiation therapy alone

Site	Fatal	Non-fatal
Bladder	5	22
Rectum	3	10
Kidney	9	1
Small bowel	2	6
Subcutaneous tissue	0	1
Bladder and adjacent structures	8	6
Total	27 (5% of 533)	46 (9% of 533)

After Miller and Johnson [8].

in the bladder. In the treatment of Stage B2, C and D1 bladder carcinoma, the generally accepted concept has been to treat the entirety of the bladder as well as generous amounts of perivesicle tissue. Miller's technique has been based on relatively large fields for the first 5000 rad, with the final 2000 rad being given with an 8×8 cm or 10×10 cm field. Present knowledge of patterns of extension of bladder cancer requires that large volumes of clinically uninvolved tissue (perivesicle and regional lymph node tissues) be irradiated to 4500–5000 rad because of the known frequency with which these tissues could be involved by sub-clinical or microscopic disease. The opportunities for reduction of volume of tissues treated to the 5000 rad level are therefore not great. *To increase the dose given to the primary site probably will depend upon techniques which will irradiate only part of the bladder.* This might well be feasible with proton beams. For example, we have considered a precision technique featuring a perineal portal with a distended bladder, illustrated in Fig. 2. A possible field arrangement and beam combination is illustrated in Fig. 3 for treatment of a lesion of the base of the bladder. A relatively standard anterior–posterior field arrangement is employed using 42 MeV X-rays to administer 5000 rad in 5 weeks to the pelvic contents (including entire bladder and rectum). Generous coverage of perivesicle and regional nodal tissue is desirable because of our limited ability to define direction and magnitude of spread

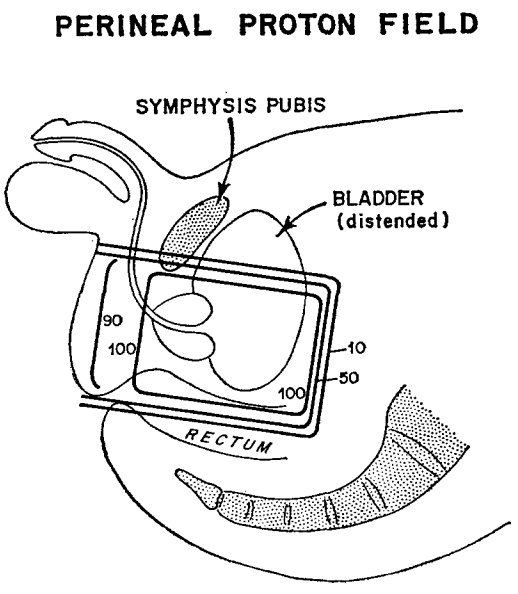


Fig. 2. Perineal proton beam portal.

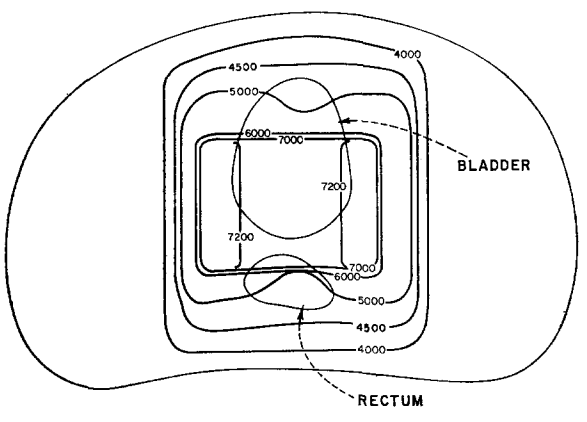


Fig. 3. Treatment plan for lesion of the base of the bladder. An anterior–posterior pair of 42 MV betatron beams are combined with a perineal proton beam.

of disease in the tissues in the *individual* patient. However, for the boost or additional dose to the clinically evident tumour there may be an important role for more sophisticated technology. The goal would be to achieve a dose distribution which is based upon the probable distribution of tumour cells. For the final 2000 rad boost to the site of the primary lesion a perineal portal, which would cover only the bladder base and anterior rectal wall, is shown. Since the bladder is distended at each treatment session, approximately one-half of the bladder would receive 5000 while the posterior half is given 7000 rad. The dose to the posterior rectal wall might be further reduced by replacing the anterior photon beam with a proton beam and precisely adjusting the proton energy so that beam penetration just reaches the anterior rectal wall (radiation sensors could be placed with advantage in the rectum to help achieve this goal.) Almost certainly this technical approach would carry a lesser morbidity, but it is not obvious that the dose to the base of the bladder could be increased from 7000 rad to 7500–7750 rad with no increase in morbidity. If not, tumour control probability would not be expected to be affected by this newer approach.

Similar considerations apply to most of the other tumour–normal tissue situations which are now being treated to near maximum dose levels.

LESIONS NOT PRESENTLY CONSIDERED FOR CURATIVE RADIOTHERAPY

Pancreas and para-aortic lymph nodes

Important clinical advances may be achieved by utilization of proton beams for treatment of lesions not now considered appropriate for radiation therapy, e.g. carcinoma of stomach, pancreas, other retroperitoneal tumours. Carcinomas of the pancreas cannot be treated to more than about 5000 rad in five weeks because of limited tolerance of the large volume of gut included in the treatment volume. With an appropriate proton beam, a dose distribution pattern whould be feasible which would encompass the pancreas with its cancer with a very narrow margin of uninvolved tissues. Treatment planning would consist of a precise, careful three-dimentional plan devised so the tumour-bearing part and

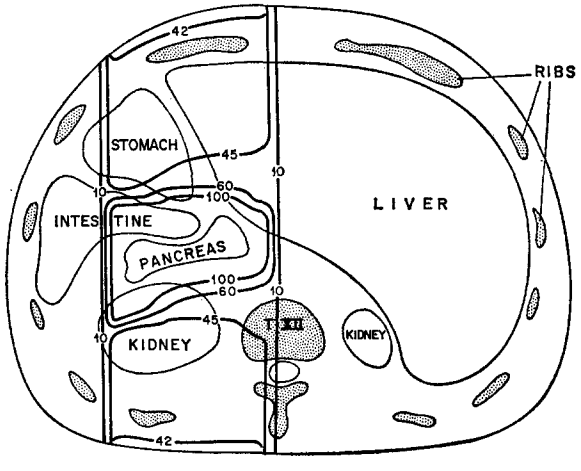


Fig. 4. Treatment plan for a pancreatic lesion using opposed modulated proton beams. Inhomogeneities are taken into account.

a minimum of adjacent bowel be irradiated (position of lesion defined by multiple clips at anterior, lateral, medial, superior and inferior extent). A sample dose distribution pattern is shown in Fig. 4. Perhaps with this type of sophisticated treatment planning the dose to a retroperitoneal carcinoma could be increased to $\geqslant 6000$ rad. If so, one would expect that, as a result of the higher dose level, more prolonged palliation or perhaps permanent control of the disease would be achieved in a worthwhile number of patients.

Here also, tolerance of a viscus which has been treated in its entirety will be a major dose-limiting factor. Treatment planning may require further refinement such as partial organ irradiation after some base line dose, say 5000 rad, has been reached.

Para-aortic lymph nodes represent another interesting target for particle therapy. At present, there is a great interest in extending the treatment portals for patients with carcinoma of the cervix, bladder, prostate and ovary to include the para-aortic lymph nodes. With available technology (anterior–posterior parallel opposed fields of 7–8 cm width) about 5000 rad in five weeks is "tolerance dose" or may exceed tolerance when combined with extensive pelvic irradiation. The limitation is due to damage to the large amount of small intestine treated by such a technique. A proton beam technique for this problem is shown in Fig. 5: a posterior proton–photon (4:1) arrangement. The marked reduction in volume of intestine included in the "tumour dose" volume would

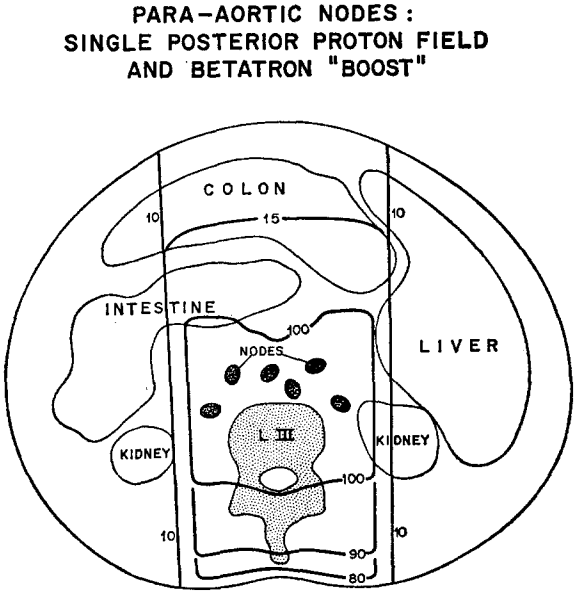


Fig. 5. Para-aortic lymph node irradiation using a posterior portal consisting of a modulated proton beam combined with a 42 MV photon beam contributing 20% of the nodal dose. Inhomogeneities are taken into account.

be expected to increase tolerance, and therefore, permit a higher dose to be given to the node-bearing region. Use of anterior–posterior pairs of carefully selected energy modulated proton beams would result in even better limitation of dose to the nodes.

There are many sites where the more idealized dose distribution patterns would be predicted to reduce frequency and sequelae of treatment even if final tumour dose were not increased over what is now administered: tumours of thyroid, many sites in the head and neck region, cervical node area, sarcoma of soft tissue (extremeties and torso) etc.

Effect of tissue heterogeneity on dose distribution for proton radiation therapy

At present there is a serious constraint in the use of particle beams because of effects of tissue heterogeneity. Both lung and bone are significant in this respect, but lung presents the greater problem. This is due both to the very low density of lung (typically, one third that of soft tissues) and the substantial thickness of lung which can be present. A 5 cm section of lung, inadvertently traversed, will extend the proton range by some 3 cm. The range distortion suffered by protons in the 100MV range when traversing bone depends on both the density and stopping power characteristics of bone and the combination leads to effective densities in the range of 1·0–1·7 relative to soft tissue. Present indications are that the range of a proton beam traversing a vertebral body would, at most, be reduced by 20% of the vertebral body thickness. Measurements are being pursued by Mr. Andy Koehler at the Harvard Cyclotron Laboratory to investigate this question.

The problem of inhomogeneities is sufficiently great that it would seem almost impossible to plan on utilizing proton beams in treatment of mediastinal structures: effects of ribs, vertebral bodies, and, most especially, air containing lung would make achievement of a good and known distribution pattern in the individual patient extraordinarily difficult. That is, assessment of distribution of dose might not be possible in a clinical setting. For sites outside of the thorax, use of protons seems not only feasible but highly attractive as a means of assessing efficacy of the radiation therapy based upon a new class of dose distribution patterns.

CONCLUSIONS

The history of radiation therapy clearly shows that when dose distribution patterns have been improved such that higher radiation

doses could be administered to the tumour-bearing site, the tumour control frequency has usually increased. Despite the improvements which have been obtained for a number of tumour–normal tissue situations by moving from orthovoltage to supervoltage irradiation, it does not necessarily follow that further improvement of dose distribution patterns will permit administration of a significantly higher dose. Total radiation dose which can be given is probably limited to something of the order of 7000–7500 rad in seven weeks. Accordingly, better results in terms of local control are not expected for those sites where doses in this range are already being employed. The improvement will be primarily in the direction of reduced morbidity.

A major area of improvement might be the treatment of sites where, by present technology, dose is limited to a substantially lower level and is unsuccessful. Reference is made here to carcinomas of the stomach, pancreas, retroperitoneal tissue, para-aortic region, thyroid, etc. Actual utilization of such beams is going to be severely hampered in certain anatomical regions because of tissue heterogeneity.

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REFERENCES

1. A. M. Koehler and W. M. Preston, Protons in radiation therapy. *Radiology* **104,** 191 (1972).
2. E. Mosekilde, Results of treatment of skin cancer with ultra soft Roentgen rays given in a single dose. *Acta Radiologica* **36,** 28 (1951).
3. E. Ebbehoj, Experience in treatment of skin cancer with ultrasoft Roentgen rays 1933. *Acta radiol.* **36,** 17 (1951).
4. A. H. W. Magnusson, Skin cancer. *Acta radiol. Suppl.* **22,** (1935).
5. E. F. VonEssen, Roentgen therapy of skin and lip carcinoma: factors influencing success and failure. *Amer. J. Roentgenol.* 556 (1960).
6. J. Nielsen, Functional results and permanence of cure following Roentgen therapy of intralaryngeal carcinoma. *J. Fac. Radiol. (Lond.)* **3,** 29 (1951).
7. R. Morrison and J. J. Deeley, The treatment of carcinoma of the bladder by supervoltage X-ray. *Brit. J. Radiol.* **38,** 449 (1965).
8. L. W. Miller and D. E. Johnson, Megavoltage irradiation for bladder cancer: alone, postoperative or preoperative? *Proc. 7th Nat'l Cancer Conf.* Lippincott, Philadelphia 771 (1973).
9. G. H. Fletcher, Supervoltage radiotherapy for cancers of the uterine cervix. *Brit. J. Radiol.* **35,** 5 (1962).

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Review of RBE Values of 15 MeV Neutrons for Effects on Normal Tissues

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Abstract—*Values of the relative biological effectiveness (RBE) of fast neutrons for effects on normal tissues depend not only on the neutron energy and the dose, but also on the type of tissue irradiated. Values of the RBE of* 15 *MeV neutrons are reviewed for rapidly proliferating rodent tissues, such as mouse bone marrow, mouse intestine and rat skin treated with single and fractionated irradiations. In addition, RBE values have been obtained for rodent tissues with relatively long cell life times, such as the vascular endothelium, the spinal cord and the lung. In general, the normal tissue data indicate that for doses between* 50 *and* 250 *rad the RBE values range up to values of about* 2, *but depend on the fractionation regimen.*

INTRODUCTION

THE INTEREST in studies of biological effects of fast neutrons in normal tissues in man and animals is related to a number of fundamental radiobiological questions. A first objective is the accumulation of information about basic mechanisms through which damage in mammalian tissues is induced and expressed. The comparison of dose–effect relations for fast neutrons and X- or gamma rays provides the possibility to analyze the influence of factors which are different for these two types of radiation, e.g. repair of sublethal damage or the oxygenation status of cells [1]. A further aim of studying neutron-induced normal tissue damage is to obtain data on the tolerance of normal tissues to single and fractionated doses, which is of importance for the application of fast neutrons in radiotherapy.

The present contribution deals with effects of monoenergetic 15 MeV neutrons, produced through the D–T reaction. This reaction offers the advantage that relatively small and inexpensive accelerators can be employed, accelerating deuterons up to 500 keV.

Values of the relative biological effectiveness (RBE) of fast neutrons for rapidly proliferating rodent tissues such as mouse bone marrow, mouse intestine and rat skin have been reported previously [2]. Over the past three years the response of these tissues has also been studied for fractionated irradiations. The introduction of higher output neutron generators has made it possible to increase the target-to-object distances, providing collimated beams, better geometry and improved dose distributions over the animal. With a modified TNC neutron generator employed at the Radiobiological Institute TNO, a neutron dose rate of 8 rad/min has been obtained at 50 cm from a new rotating tritium target, bombarded with 270 keV deuterons at a current of 6 mA.

Quantitative studies have recently been initiated on the responses of tissues with relatively long turnover times, such as the vascular endothelium, the spinal cord and the lung. The effects of irradiation on these slowly proliferating cell-renewal systems, which are frequently dose-limiting in clinical radiotherapy, may appear only after long observation periods of up to one year after exposure. Some late effect studies on slowly proliferating tissues after fast-neutron irradiation will be summarized.

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RBE VALUES FOR EFFECTS ON RAPIDLY PROLIFERATING TISSUES AFTER SINGLE-DOSE IRRADIATION

It has been reported previously [2] that the RBE values of 1·1 for bone marrow syndrome and 1·4 for intestinal syndrome are in agreement with the results obtained for the survival of haemopoietic stem cells and the intestinal crypt stem cells, respectively. The RBE of 1·4 for the survival of jejunal crypt cells has also been obtained independently by Withers *et al.* [3]. The RBE values for effects on haemopietic stem cells are also significantly lower for larger animals; an RBE of 1·02 was reported for the 30-day mortality of dogs after irradiation with 14·6 MeV neutrons [4]. As discussed in detail elsewhere [1] the differences in RBE values for the two radiation syndromes are due to intrinsic differences in radiosensitivity between the bone marrow stem cells and the intestinal crypt stem cells. Recently, higher RBE values of 1·6 have been reported, both for the $LD_{50/30d}$ and for survival of colony forming units (CFU) in mice [5,6]. The larger RBE values might be attributed to differences in the quality and characteristics of the animals employed in the studies or to differences in the neutron dosimetry and in the energy spectrum of the fast neutron beams employed. The general tendency of increasing RBE with decreasing neutron energy, initially shown for cultured cells, has been observed for the occurrence of the bone marrow syndrome as well as for the intestinal syndrome, as can be seen from Fig. 1.

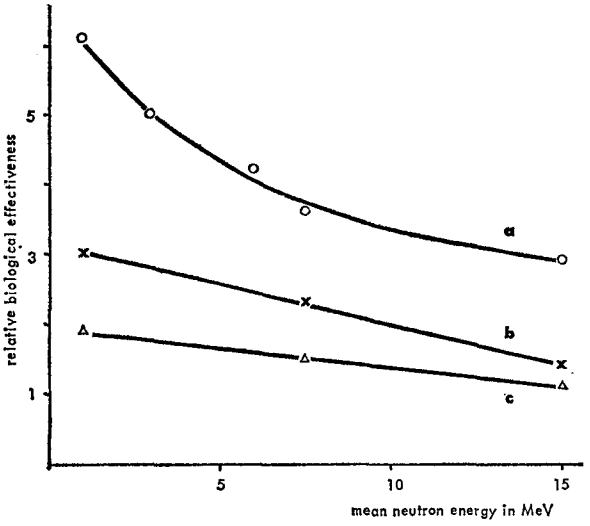


Fig. 1. *RBE as a function of mean neutron energy for survival of cultured cells at low doses, producing* 20% *cell reproductive death (curve a) and for induction of intestinal syndrome (curve b) and haemopoietic syndrome (curve c) in mice. The results obtained for the two radiation syndromes by Hornsey* et al. [7] *and Davids* [8] *for cyclotron neutrons and fission neutrons, are included in the figure.*

An example of the effect of the quality of the neutron beams is provided by the studies on CFU survival in mouse bone marrow carried out at Manchester and Rijswijk [9]. The methods and materials used by the two groups were very similar, but the biological results indicated a 25% greater effectiveness for the fast neutron beam employed at Manchester. The *in vivo* irradiations of the animals at Rijswijk were carried out free-in-air, whereas the Manchester irradiations were performed

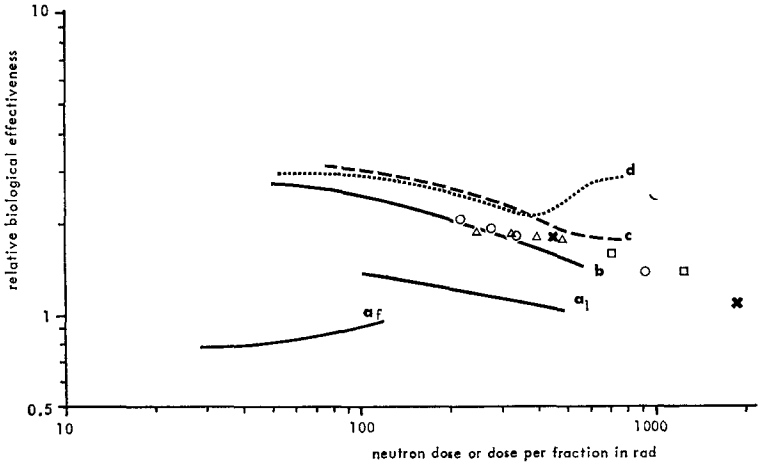


Fig. 2. *Relations between the RBE of* 15 *MeV neutrons and the neutron dose or daily dose per fraction for mouse intestinal crypt cells* (○), *rat capillary endothelium* (△), *rat skin* (□), *mouse haemopoietic stem cells after single doses (curve* a_1*) and after* 5 *fractions given at daily intervals (curve* a_f*), cultured cells of human kidney origin (curve b), mouse osteosarcoma (curve c) and rat rhabdomyosarcoma (curve d).*

inside a steel collimator. It has been demonstrated that under the latter conditions 20% of the neutron dose is delivered by scattered neutrons. The contribution of these low energy neutrons will tend to increase the RBE. A similar increase in RBE values has been found by Hendry *et al.* [10] for the survival of intestinal crypt stem cells. RBE values between 1·8 and 2·0 are reported which are higher than the value of 1·4 as observed by other groups for free-in-air irradiations.

RBE VALUES FOR EFFECTS ON RAPIDLY PROLIFERATING TISSUES AFTER FRACTIONATED IRRADIATIONS

The results after single-dose irradiation have provided basic information on survival curve parameters for the various types of tissues; however, in view of the clinical applications, the RBE values for fractionated irradiations are of greatest importance. In general, the repair of sublethal damage, associated with the presence of a shoulder in the survival curve, is larger for X-irradiation than for fast-neutron irradiation. Consequently, for the same total doses higher RBE values are observed for fractionated irradiation than for single-dose irradiation. Experimental data on the survival of jejunal crypt stem cells are obtained at relatively large doses. Extrapolation to low dose values can be obtained from a comparison of the effects for single and fractionated irradiations [11]. On the basis of these results it can be concluded that the minimum values of the D_Q are equal to 130 rad and 340 rad for the neutron and X-ray survival curve, respectively. The resulting curves for neutrons and X-rays can be characterized by D_0 values of 140 rad and 167 rad and extrapolation numbers of 2·6 and 7·6, respectively. Studies of the survival of jejunal crypt stem cells after irradiation with 5 daily fractions have yielded RBE values of 2·1, 1·95 and 1·85 for neutron doses of 220, 280 and 340 rad per fraction, respectively [11]. These values can be compared with the RBE of 1·4 obtained at a neutron dose of 920 rad for a single exposure.

Studies on spleen CFU survival in the mouse after fractionated irradiation showed a slightly higher effectiveness for X-rays than for fast neutrons, i.e. the RBE of 15 MeV neutrons is smaller than 1·0 [12]. This result is unexpected in view of the small shoulder in the survival curve for single-dose irradiations with 15 MeV neutrons and the smaller repair which is usually observed in split-dose neutron irradiations in comparison with X-rays. A partial explanation for this observation can be deduced from another series of experiments studying the effects of single doses of X-rays and fast neutrons *in vivo* whereby a delay of 24 hr was allowed between irradiation and sacrifice of the donors for assay. The delayed assay causes a marked reduction in 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. This finding has been confirmed in later studies of Hendry and Howard [13]. These phenomena imply that the RBE value for immediate assay is higher than the RBE for delayed assay and for fractionated exposures. The loss of CFU after X-irradiation has been described by a number of investigators. At present, it seems most likely that this loss is due to differentiation of colony-forming cells to a cell type which has lost the capacity for proliferation to the size of a visible spleen colony. However, so far there is no explanation for the absence of this phenomenon after neutron irradiation. The studies on CFU survival are included in the present review to emphasize that in addition to differences in repair of sublethal damage for X- and neutron irradiations, differences in other biological mechanisms will determine the overall effectiveness after fractionated exposures. The interpretation of some of these qualitative differences will require additional information on the population kinetics of the stem cells after X- and neutron irradiation.

RBE VALUES FOR EFFECTS ON SLOWLY PROLIFERATING TISSUES

Radiation-induced late changes in a number of normal tissues, e.g. lung, kidney and heart, might be attributed to damage of the blood vessels. An experimental method which enables a direct quantitative measurement of vascular proliferation in subcutaneous tissue of the rat has recently been developed by Reinhold and Buisman [14]. This technique in which capillaries are induced to proliferate in a two-dimensional system has been employed to study the radiosensitivity of capillary endothelium to irradiation with X-rays and 15 MeV neutrons. The assay is performed in the upper layer of the subcutis of the back of the animal which is distended from the underlying tissue by a subcutaneous injection of air. A small area is depleted of blood vessels by a freezing procedure with liquid nitrogen. Before irradiation of the area, a stimulus is applied to induce vascular proliferation [14]. Quantification of the effects

on the proliferating capacity of the endothelium is performed with an angiographic technique, employing microfluorometry. The extent of revascularization in the test area has been taken to represent the survival of the capillary endothelium.

Dose–effect curves have been obtained for single-dose irradiations with collimated beams of 300 kV X-rays and 15 MeV neutrons. From the survival curves D_0 values of 170 rad and 95 rad and extrapolation numbers of 7 and 5 can be derived for X-rays and 15 MeV neutrons, respectively. For surviving levels between 40 and 4%, corresponding to neutron doses between 250 rad and 500 rad, respectively, the RBE varies only slightly from 1·9 to 1·8. It can be concluded that the radiosensitivity of the endothelial cells of capillaries for X-irradiation is of the same magnitude as that of epithelial cells and cells of supporting tissues, and that the RBE values for effects of 15 MeV neutrons on the capillary endothelium are also similar to the RBE values obtained so far for other types of normal tissues, e.g. skin and intestinal epithelium.

Quantitative data concerning effects of fast neutrons on the central nervous system are very scarce. Investigations of damage to the spinal cord after irradiation with X-rays and 15 MeV neutrons have been carried out by Van der Kogel and Barendsen [15] with WAG/Rij rats, using muscle atrophy and paralysis of the hind legs as a measure of the induction of myelopathy. Depending on the dose and dose fractionation schedule, palpable muscle atrophy and impaired use of the hind legs were observed after a minimum latent period of about 3 months [15]. Total paralysis resulted after exposure with larger doses. The lowest single doses causing observable motor damage within one year were 2000 rad of 300 kV X-rays or 1900 rad of 15 MeV neutrons. Equivalent doses administered in five fractions with 24 hr intervals were 4000 rad of 300 kV X-rays and 2250 rad of 15 MeV neutrons. From these data, RBE values of 1·1 and 1·8 were derived, respectively. These values are not significantly different from the RBE values obtained with similar doses or dose fractions for damage to skin and intestinal tract. However, a marked qualitative difference was noted between the effects of X-rays and 15 MeV neutrons since after irradiation with 15 MeV neutrons there is a shorter latency period and, once initiated, a more rapid progression of the muscle atrophy.

Lung tissue, which has a very slow cell proliferation of the capillary endothelium, is one of the dose-limiting tissues for irradiations of the thoracic region. According to the studies of Phillips and Margolis [16], for the irradiation of the thorax of mice, the mortality between 40 and 180 days can be taken as an end-point for pulmonary damage. The time period in which mortality can occur, will be different for various mouse strains. In studies with CBA mice at Rijswijk in cooperation with Hollander, mortality is generally found about 200 days post-irradiation. Histological studies of the radiation-induced lung damage have shown late radiation fibrosis which appears to be related to capillary injury in the alveolar septa. The process is characterized by a distinct thickening of the septa and a reduction of the alveolar air spaces.

Preliminary results indicate an RBE of about 1·2 for the $LD_{50/210d}$ for a single-dose irradiation with 1200 rad of 15 MeV neutrons. This RBE value is slightly lower than the RBE values obtained for epithelial tissues in this range of neutron doses.

COMPARISON OF RBE VALUES FOR EFFECTS ON NORMAL TISSUES AND TUMOURS

The results discussed clearly indicate that a considerable variation of intrinsic radiosensitivities exists for X-rays, bone marrow stem cells being the most sensitive and intestinal crypt stem cells being very resistant. Furthermore, it can be concluded that similar but somewhat smaller differences are observed for irradiations with 15 MeV neutrons.

The experimental data on the RBE values of fast neutrons for effects on various normal tissues have been summarized in Fig. 2. RBE values have been presented as a function of the dose or dose per fraction. The data show a general tendency for an increase of the RBE with decreasing dose or dose per fraction, which is to be ascribed to the smaller influence of accumulation of damage after neutron irradiation. In this figure, RBE values are also included for effects on two types of animal tumours.

For the application of fast neutrons in radiotherapy, the RBE values in the dose region between 50 and 250 rad are presumably the most important, because with very small fractions, too large a number of fractions and too long overall times would be required, whilst dose fractions in excess of 250 rad would be expected to be too large to be tolerated by various normal tissues. It can be concluded from a comparison of the curves for tumours with the curves for various normal tissues, that

in the range of doses between 50 and 250 rad the overall gain factor $RBE_{tumour}/RBE_{normal\ tissue}$ does not exceed a factor of 1·2–1·3 [17]. This might still provide a significant advantage in clinical applications if the dose distributions in the patient will be equivalent to those obtained with high energy X-rays. In addition, because of the reduced influence of accumulation of damage and of hypoxia of cells, it might be speculated that the effectiveness of fast neutrons for treatments of tumours might be more reproducible, that this effectiveness would be less dependent on the fractionation regimen employed, and consequently, that treatments can be adapted to various growth properties of tumours and the dose-limiting normal tissues involved.

Acknowledgements—The experimental data from the Radiobiological Institute TNO summarized in this paper represent the joint efforts with a number of colleagues: G. W. Barendsen, C. F. Hollander (Institute for Experimental Gerontology TNO), A. J. van der Kogel, L. M. van Putten and H. S. Reinhold. The contributions to the experimental investigations and the stimulating discussions are gratefully acknowledged.

REFERENCES

1. J. J. Broerse and G. W. Barendsen, Relative biological effectiveness of fast neutrons for effects on normal tissues. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard), Vol. 8, p. 305. North-Holland, Amsterdam (1973).
2. 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).
3. 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).
4. J. D. Earle, E. J. Ainsworth and G. F. Leong, Lethal and hematologic effects of 14·6 MeV neutrons on beagles with estimation of RBE. *Radiat. Res.* **45,** 487 (1971).
5. H. Langendorff, M. Langendorff, R. Metzner, H. Mönig, K.-H. Steinbach and G. Tumbrägel, Radiobiological investigations with fast neutrons. I. Comparative investigations on the mortality of male mice after an irradiation with 15 MeV neutrons and ^{60}Co γ-rays. *Atomkernenergie* **16,** 255 (1970).
6. T. L. Phillips, H. H. Barschall, E. Goldberg, K. Fu and J. Rowe, Comparison of RBE values of 15 MeV neutrons for damage to an experimental tumour and some normal tissues. *Europ. J. Cancer* **10,** 827 (1974).
7. S. Hornsey, S. Vatistas, D. K. Bewley and C. J. Parnell, The effect of fractionation on 4-day survival of mice after whole-body neutron irradiation. *Brit. J. Radiol.* **38,** 878 (1965).
8. J. A. G. Davids, Bone-marrow syndrome in CBA mice exposed to fast neutrons of 1·0 MeV mean energy. Effect of syngeneic bone-marrow transplantation. *Int. J. Radiat. Biol.* **17,** 173 (1970).
9. J. J. Broerse, A. C. Engels, P. Lelieveld, L. M. van Putten, W. Duncan, D. Greene, J. B. Massey, C. W. Gilbert, J. H. Hendry and A. Howard, The survival of colony-forming units in mouse bone-marrow after *in vivo* irradiation with D–T neutrons, X- and gamma-radiation. *Int. J. Radiat. Biol.* **19,** 101 (1971).
10. J. H. Hendry, D. Major and D. Greene, In Discussion on dosimetry and collimation of fast-neutron beams. B. Biological aspects of dosimetry. *Europ. J. Cancer* **10,** 325 (1974).
11. J. J. Broerse and H. Roelse, Survival of intestinal crypt cells after fractionated exposure to X-rays and 15 MeV neutrons. *Int. J. Radiat. Biol.* **20,** 391 (1971).
12. L. M. van Putten, P. Lelieveld and J. J. Broerse, Effects of 300 kV X-rays and 15 MeV neutrons in single and fractionated doses on hemopoietic stem cells in mouse bone marrow. In *Proc. Conf. Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, p. 162. Carmel, Calif., BNL Publ. nr. 50203 (C-57), (1970).
13. J. H. Hendry and A. Howard, The response of haemopoietic colony-forming units to single and split doses of γ-rays or D–T neutrons. *Int. J. Radiat. Biol.* **19,** 51 (1971).
14. H. S. Reinhold and G. H. Buisman, Radiosensitivity of capillary endothelium. *Brit. J. Radiol.* **46,** 54 (1973).

15. A. J. VAN DER KOGEL and G. W. BARENDSEN, Late effects of spinal cord irradiation with 300 kV X-rays and 15 MeV neutrons. *Brit. J. Radiol.* (1973) (In press).
16. T. L. PHILLIPS and L. MARGOLIS, Radiation pathology and the clinical response of lung and esophagus. In *Frontiers of Radiation Therapy and Oncology* (Edited by J. M. VAETH), Vol. 6, p. 254. Karger, Basel (1972).
17. G. W. BARENDSEN, Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. *Europ. J. Cancer* **7**, 181 (1971).

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The RBE of Cyclotron Neutrons for Effects on Normal Tissues

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Abstract—*The RBE for damage to various normal tissues is shown to be dependent on the tissue at risk and on the dose per fraction used. At present there is no method of predicting the RBE for any tissue.*

INTRODUCTION

THE RBE OF fast neutrons, when measured for tissue reactions, and plotted as a function of dose per fraction is in accordance with that which could be predicted from the shapes of typical survival curves for cells irradiated with X-rays or neutrons. For small doses, in the "shoulder region" of the survival curves, the RBE is high due to the smaller "shoulder" of the neutron curve. With increasing dose the RBE decreases, as the effect of the difference in shoulder size for neutrons and X-rays diminishes, and tends to the ratio of D_0 for X-rays to that for neutrons. RBE curves of this type have been presented for a number of different normal tissues, by Field and Hornsey, for neutrons from the cyclotron at Hammersmith [1]. From these observations it was concluded (a) that the shape of the RBE curve was similar for different tissues (b) that there are significant differences in the absolute values of RBE for different tissues as a function of dose per fraction, (c) that at least for skin reactions the RBE was the same for all four species observed, i.e. man, pig, mouse and rat, and (d) observations on rat feet and pig skin suggest that the RBE values for late reactions in skin are very similar to those for the early reactions at the same dose per fraction.

The present communication brings up to date the observations made on the tissues previously discussed and presents data for two other tissues, i.e. lung and oesophagus.

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Skin

Data obtained since the previously published curve on skin response are in good agreement with the early data [1]. A recent value of RBE for the response of human skin, derived from skin reactions in patients who had tumours irradiated with 12 fractions of neutrons or X-rays, falls on this early curve [2], as do RBE values obtained from experiments on mouse skin given single doses, five or nine fractions [3] and fifteen fractions (Field unpublished).

After irradiation of the feet of rats, there is an early skin reaction followed by a late wave of damage, leading to deformity. Mice feet do not show the late wave of skin reaction, but do become deformed. Deformity of mice feet was scored in the fifteen fraction experiments and for single doses. The relationship between deformity and early reaction was the same for both fractionation regimes with both X-rays and neutrons, and was very similar to that shown for rat feet [1].

An "Ellis Formula" for fast neutrons

In order to adjust the dose of X-rays, in radiotherapy, in a known and consistent way with changes of fraction number and overall treatment time, Ellis [4] suggested the following empirical relationship between total dose, (TD), overall treatment time (T days) and number of fractions (N) to be

$$TD = NSD_x N^{0.24} T^{0.11}$$

where NSD_x is the nominal standard dose for X-rays. A similar formula has been derived for fast neutrons based on data from animal and human skin reactions and assuming the exponent for T remains the same (i.e. assuming repopulation is the same for X-rays and neutrons)

$$\text{i.e. } TD = NSD_N \, N^{0.04} \, T^{0.11}$$

where NSD_x is the nominal standard dose for fast neutrons [5]. The exponent for N is reduced for neutrons because of the smaller capacity of skin to repair sublethal damage after neutron irradiation. This empirical formula for fast neutrons is currently used to adjust the neutron doses when the standard treatment regime is altered in the treatment of patients with the neutron beam at Hammersmith.

Intestine

The response of mouse intestine to X-rays is complicated by a marked effect of dose rate [6] but it has been shown recently [7] that at the same dose rate, the RBE for 8 MV X-rays relative to 250 kVp X-rays is not significantly different from 1. Therefore all the gut RBE data from Hammersmith, for both 8 MV and 250 kVp X-rays, has been included in an analysis of RBE. A recent experiment has been performed to measure the RBE for intestinal damage at the dose levels currently employed in radiotherapy. This was obtained by giving four fractions at time intervals of 12 hr and using the surviving of crypts as the endpoint. The RBE obtained was 3·8 at 140 rad per fraction of neutrons, compared with 3·0 for skin at the same dose level, which is consistent with RBE values for gut damage being about 20% higher than for skin [1].

Cartilage and haemopoietic tissues

No new data have been obtained for these tissues.

Lung and oesophagus

It was shown by Phillips and Margolis [8] that after irradiation of the thorax only, mice die either in the first month, or may survive for about 3 months and then die. We have repeated these experiments, irradiating with X-rays or neutrons, and confirm this pattern of death after both radiations.

For the smaller dose in Fig. 1, and in general for less than about 1000 rad of neutrons or 2500 rad of X-rays, animals survived until about day 80, and those still surviving at day 160 appeared to have a life expectancy similar to that of unirradiated mice. Histological examination showed that the animals which died between 80–160 days had marked lung damage; there were areas of consolidation, increased cellularity, chronic inflammation and signs of developing fibrosis. Phillips [9], from observations on mice, concluded that such animals died from pneumonitis but that the primary damage was to the capillary endothelial cells which break down and block the capillaries. Maisin [10] in a study of radiation pneumonitis concluded that direct damage to the parenchymal cells contributed to the pneumonitis as well as indirect effects on the parenchymal secondary to the injury to the vasculature [8]. In the same dose range, Kurohara and Casarett [11] have investigated the death of rats after irradiation of the thorax, and considered death was due to pneumonic infection, superimposed on a developing pulmonary fibrosis. They also found a significant reduction in the partial

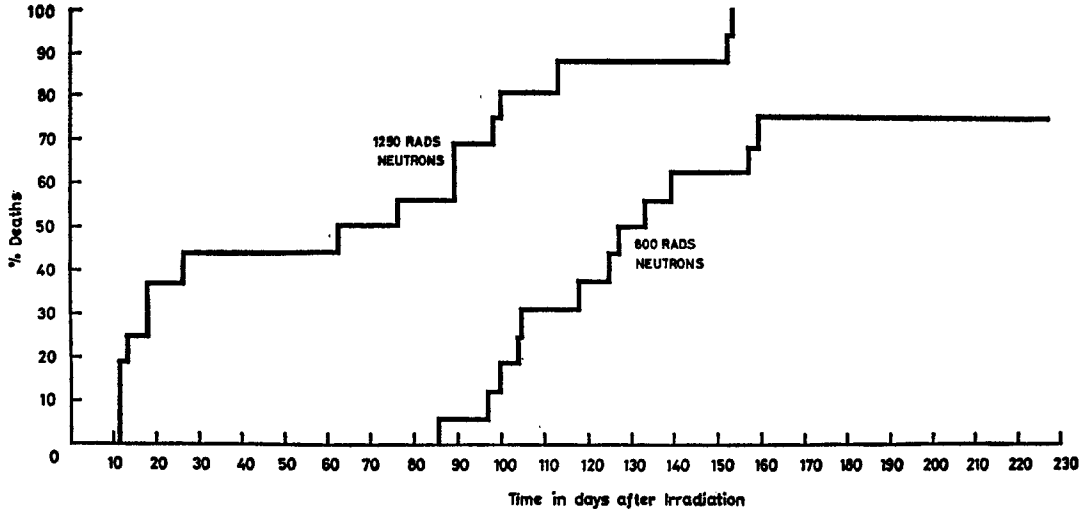


Fig. 1. The proportion of dead mice as a function of time after irradiation of the thorax.

pressure of oxygen in arterial blood at 120 days post irradiation.

After larger doses, i.e. above 1000 rad of neutrons or 2500 rad of X-rays, mice die between day 10 and day 30, with evidence of breakdown of the oesophageal epithelium and loss of the keratinized layers. Phillips and Margolis [8] suggest that this earlier thoracic damage is the result of the loss of basal cells in the epithelium of the oesophagus. Kurohara and Casarett [11] confirm this observation in the rat, and associate death at this time with a marked reduction in food intake and body weight. It is seen in Fig. 1 that mice which survive 30 days do generally continue to do so until they may die of pulmonary damage. At this time the oesophageal mucosa generally appears completely normal. In our experiments a few mice died during the first week after the highest doses used (above about 3500 rad of X-rays in a single dose, or equivalent with neutrons), due to damage to an intestinal loop included in the irradiation field.

For assessing the RBE we have used two endpoints: (a) LD/50 between 10 and 30 days (oesophageal damage) and (b) LD/50 between 40 and 180 days (pulmonary damage). For the two endpoints, the irradiation procedures and radiation geometry were identical; only the dose levels were different. The RBE values for single doses and two fractions with an interval of 24 hr for oesophageal damage were at least 30% *greater* than for skin at the same dose per fraction, whilst the RBE values for lung damage were at least 25% *less* than for skin. This situation is roughly analagous with that seen after whole-body irradiation where the RBE for LD/50 in 5 days (intestinal damage) is far greater than the LD/50 in 30 days (haemopoietic damage).

DISCUSSION

Figure 2 shows the relationship between RBE and dose per fraction for the normal tissues mentioned above. For clarity, the data points have not been included except for the RBE values for lung and oesophageal death. The curve for skin is now very well defined, and after the addition of new data remains hardly different from that presented by Field and Hornsey [1]. The curve for intestine has been extended to lower doses and the RBE remains significantly higher than for skin. No new data are available for catilage and haemopoietic tissues, the curves are those previously published. The RBE for 1 and 2 fractions to lung is low, as are also the values for haemopoietic tissues. The explanation for the low RBE haemopoietic tissues is that there is very little recovery from sublethal damage after X-rays or neutrons and the RBE largely reflects the ratio of the D_0 values. For both lung and oesophageal damage, however, there is a considerable degree of recovery from sublethal damage after both X-rays and neutrons. The relative recovery after neutrons compared with X-rays allowing for the RBE, derived from:

$$\frac{D_2-D_1}{D_1} \text{ for neutrons} \qquad \frac{D_2-D_1}{D_1} \text{ for X-rays,}$$

is close to 1 in both cases. This value is greater than for skin or gut, and providing the lung

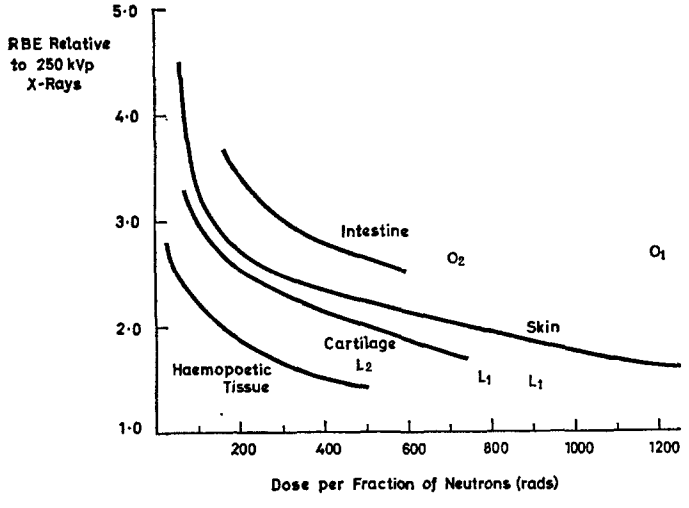


Fig. 2. RBE of Hammersmith cyclotron neutrons for different tissues, as a function of dose per fraction. O_1 and O_2 represent values for single doses and two fractions for oesophageal death, L_1 and L_2 represent values for lung death.

and oesophageal damage is cellular in origin, the implication is that the survival curve is only slightly altered in shape by the more densely ionizing radiation. Thus, as for haemopoietic tissues, the RBE is largely determined by the ration of D_0 values which presumably is large for oesophagus and small for lung.

SUMMARY AND CONCLUSIONS

The RBE for different normal tissues, as a function of dose per fraction is variable, the maximum differences being a factor of nearly 2. The reasons for these differences in RBE are the different characteristics of the survival curves of the various cells when irradiated with X-rays or neutrons, but there is not, as yet, any way to predict the RBE. It remains to be shown for tissues other than skin, that the RBE in man is the same as in mouse, but if true, measurements such as those summarized in Fig. 2 will be an essential part of any radiotherapy programme involving a new type of radiation.

REFERENCES

1. 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).
2. M. Catterall, C. Rogers, R. H. Thomlinson and S. B. Field. An investigation into the clinical effects of fast neutrons. *Brit. J. Radiol.* **44,** 603 (1971).
3. J. F. Fowler, J. Denekamp, A. L. Page, A. C. Begg, S. B. Field and K. Butler. Fractionation with X-rays and neutrons in mice: response of skin and C_3H mammary tumours. *Brit. J. Radiol.* **45,** 237 (1972).
4. F. Ellis, Time, dose and fractination: a clinical hypothesis. *Clin. Radiol.* **20,** 1 (1969).
5. S. B. Field, The Ellis formulae for X-rays and fast neutrons. *Brit. J. Radiol.* **45,** 315 (1972).
6. S. Hornsey and T. Alper, Unexpected dose rate effect in the killing of mice by radiation. *Nature (Lond.)* **210,** 212 (1966).
7. S. Hornsey and A. Silvester, The relative effectiveness of super- and orthovoltage X-rays for intestinal damage in mouse and man: an apparent discrepancy and its explanation. *Brit. J. Radiol.* **46,** (In press) (1973).
8. T. L. Phillips and L. Margolis, Radiation pathology and the clinical response of lung and oesophagus, In *Frontiers of Radiation Therapy and Oncology*, Vol. 6 p. 254 Karger, Basel, (1972).
9. T. L. Phillips, An ultrastructural study of the development of radiation injury in the lung. *Radiology*, **87,** 49 (1966).
10. J. R. Maisin, The ultrastructure of the lung of mice exposed to a supralethal dose of ionizing radiation on the thorax. *Radiat. Res.* **44,** 545 (1970).
11. S. S. Kurohara and G. W. Casarett, Effects of single thoracic X-ray exposure in rats. *Radiat. Res.* **52,** 263 (1972).

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Cellular Repair After X or Gamma Irradiation Determined for Several "Gross" Criteria on Animals and Patients*

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Abstract—*The shape of survival curves for several mammalian cell types has been studied by assessing the "recovered dose" (early recovery) for different sizes of the fractions. The following* "gross" end-points *have been used: skin reactions and intestinal death in mice, tumour regression, gross skin and mucosal reactions in patients.*

The increase of dose necessary to attain the chosen biological effect has been determined when a single irradiation dose D_s *is split* into two equal doses $2D_i$ *or when* N *fractions* $(N \times D_s)$ *are "split" into* 2N equal *"subfractions"* $(N \times 2D_i)$; i *is the interval between the two "subfractions"* D_i *and the overall treatment time is kept constant.*

From the data obtained conclusions are drawn about the shape of cell survival curves in the low dose region and some implications for fast neutron therapy are discussed.

INTRODUCTION

FOR SEVERAL years, research work has been carried out to evaluate quantitatively cellular repair (Elkind repair) as a function of the dose per fraction, especially for the relatively small doses per fraction currently used in radiotherapy. Several "gross" tissue reactions have been used as criteria: intestinal death in mice, skin reactions in mice, skin reactions in patients, tumour regression in patients.

The first part of this paper deals with experimental methods and results. In the second part, some consequences of our results related to the shape of cell survival curves and some implications in neutron therapy, particularly with respect to fractionation will be discussed.

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EXPERIMENTAL METHODS AND RESULTS

A. Rationale of the experiments

Additional dose necessary to reach a given biological effect has been assessed when a dose D_s is split into two equal fractions $2D_i$ separated by a time interval i. For example, our data show that if intestinal death in mice (see below) is taken as the biological criterion, the LD_{50} value increases from 1005 rad to 1290 or 1440 rad when a single dose is split into two equal fractions separated by an interval $i = 3{\cdot}5$ or 24 hr respectively. The dose increment $2D_i = D_s$ (in this case the difference of LD_{50}) will be designated by D_r. If we want to study smaller doses per fraction and to retain the same gross end-point (LD_{50} for intestinal death), we have to repeat the irradiation N times, depending on the size of the fractions. Thus for small doses per fraction, such as those usually given in radiotherapy,

we have to compare two irradiation protocols A and B, having the *same overall time* but in which each fraction D_s in protocol A is replaced in protocol B by two *equal* "subfractions" $2D_i$ separated by an interval i (Fig. 1). If the final biological effect is the same for irradiation protocols A and B, we can assume that the effect of one fraction D_s is equal to the effect of the *corresponding* two "subfractions" $2D_i$. The difference $D_r = 2D_i - D_s$ can be ascribed to a difference in cell repair (Elkind type repair [1]) when a dose D_s is split into two equal "subfractions" $2D_i$ and will be called: "*differential recovered dose*" for cell repair. This point and particularly the choice of a suitable interval i have already been discussed [2,3].

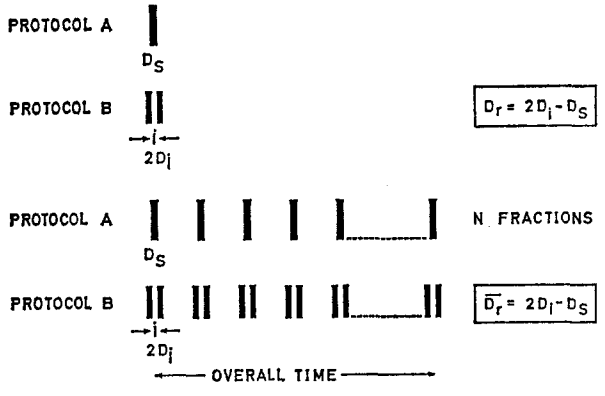
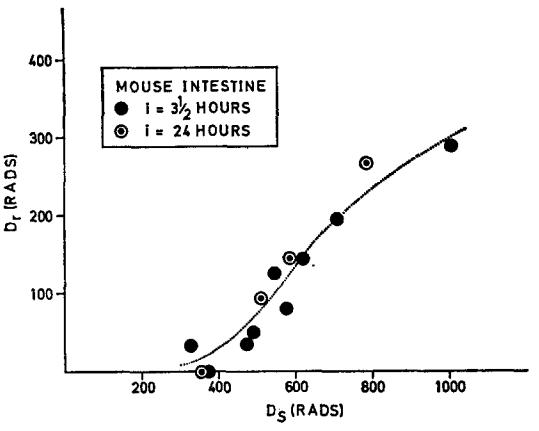


Fig. 1. Scheme of the experiments with fractionated irradiation.

B. Experimental results

(1) "*Intestinal death*" *in mice.* Our first criterion is "intestinal death" in mice scored 20 or 30 days after irradiation of the abdomen. The abdomen only was irradiated, the rest of the body being shielded in particular in order to avoid the bone marrow syndrome [3]. The intestinal lesions have been studied by systematic histological examination (in collaboration with J. R. Maisin, to be published). Irradiations consisting of $N = 1, 2, \ldots$ up to 10 daily fractions have been performed. For each irradiation protocol ($N \times D_s$ or $N \times 2D_i$), a complete dose–survival curve has been determined, from which the values of LD_{50} (and their differences) could be derived. Our results obtained for "intestinal death" in mice are summarized in Fig. 2, where "differential recovered dose" ($D_r = 2D_i - D_s$) is expressed as a function of the dose per fraction D_s. D_r is very small for the usual doses per fraction in radiotherapy; it increases when the dose per fraction increases and reaches 70 rad for $D_s = 500$ rad and 200 rad for $D_s = 700$ rad.

It should be pointed out that a small value of D_r does not mean that there is no (or little) cellular recovery (Elkind type recovery) *but that the difference in cellular repair is small between the compared protocols.* Irradiations have been performed with intervals $i = 3{\cdot}5$ or 24 hr between the subfractions (for protocol B). The results are quite similar for these two considered intervals i.



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Fig. 2. Differential recovered dose D_r *is plotted against dose per fraction* D_s. *This figure summarizes results obtained for intestinal death in mice: the closed circles correspond to an interval* i = 3·5 *and the open circles to an interval* i = 24 *hours between the subfractions* $2D_i$.

(2) *Skin reactions in patients.* The second biological criterion is the evaluation of skin reactions in patients. The methodological approach has already been described in detail elsewhere [2]. Skin reactions observed in the same patient on two symmetrical fields irradiated with two different protocols are compared. As for the first criterion, the effect of a dose D_s and of 2 doses $2D_i$ separated by a time interval i are compared. Depending on the size of the dose fraction, they are repeated N times to reach *dry desquamation.* The overall time is always the same for the two compared protocols.

The reactions are scored according to the degree of desquamation, but more important than the score itself is the clinical judgement of a *clear difference* between the reactions on the two fields which allowed assessment of an upper and lower limit of the values of D_r as a function of D_s.

The results obtained for skin reactions for 77 patients so far observed are shown by the hatched area of Fig. 3; this hatched area can be considered as the confidence interval of D_r [2]. Thirty patients have been treated with an interval of 6 hr between the subfractions; 47 with an interval $i = 24$ hr (this is equivalent to comparing daily fractionation and irradia-

tion every other day). Similar to results for "intestinal death" in mice, no significant difference was observed in the values of D_r as a function of D_s related to the interval i for $i=6$ or 24 hr [3].

Figure 3 shows that the results obtained for human skin do not differ very much from those obtained for "intestinal death" in mice.

(3) *Skin reactions in mice.* Experiments using skin reactions in mice as biological criterion have been described elsewhere [4]. The results summarized in Fig. 3 are at the upper limit of those obtained for human skin reactions and "intestinal death" in mice.

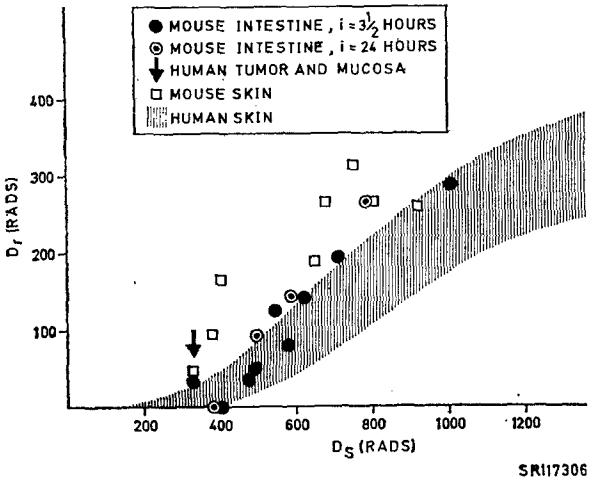


Fig. 3. Comparison of the variation of D_r *as a function of* D_s *for different biological criteria: intestinal death in mice, tumour regression and mucosal reactions in patients (see text), skin reactions in mice and skin reactions in patients (hatched area). The observed values of* D_r *as a function of* D_s *do not differ very much for the considered end-points. The doses are normalized for* ^{60}Co.

(4) *Tumour regression and mucosal reactions in patients.* A clinical trial (Institut Gustave-Roussy, unpublished) was carried out on patients with epithelioma of the tonsil to compare the effect of 14 sessions of 330 rad in 32 days with 14 split sessions of 2×190 rad in 32 days, with a time interval of 2 hr between the subfractions. Tumour regression was faster for the "split" irradiation (protocol B) than for the conventional fractionation (protocol A); however, the mucosal reactions were also slightly more significant. This indicates that the value of D_r at 2 hr is smaller than 50 rad and there was no evidence of any differential effect between the tumour regression and the mucosal reaction associated with the subfractionation. Assuming the same kinetics as for the other tissues studied, it may be accepted that D_r at 6 or 24 hr is less than 65 rad for $D_s=330$ rad (arrow pointing downwards in Fig. 3). However, the tumour regression is of only little significance as a biological test and a longer follow-up period is required for conclusions to be drawn on the effect of subfractionation on the cure rate [5].

DISCUSSION AND CONCLUSIONS

A. Importance of the numbers of fractions in practical radiotherapy

First of all, *without any hypothesis,* some conclusions may be derived directly from our experimental results. These conclusions are mainly relevant directly to radiotherapy with X- or gamma rays.

(1) In a fractionated treatment, the total dose corresponding to a given effect does not depend very much on the number of fractions, provided that the *overall time is kept constant,* and that the *doses per fraction are not larger than* 300 *rad.* For larger doses per fraction, the increase of the total dose with increasing number of fractions depends on the size of the fractions. One can derive from Fig. 2 that if N fractions of 400 rad are needed to reach a given effect, and if these N fractions are split into $2N$ "subfractions", one has to add 30 rad per fraction (i.e. about 7%). On the other hand, if fractions of 700 rad are split into equal subfractions, one has to add 200 rad per fraction (i.e. about 30%).

(2) Because the curves D_r vs D_s are rather similar for the tissues which have been investigated (Fig. 3), *a significant differential effect related to fraction number* is unlikely between these tissues in the usual conditions encountered in radiotherapy. A similar conclusion has been derived by Withers [6] from the comparison of the data in the literature concerning different types of normal and cancer tissues or cell lines. However, a significant differential effect related to *overall time* could be expected. In fact, repopulation seems to be more important for intestinal stem cells than for skin [7,8].

B. Direct determination of the shape of cell survival curves from gross tissue reactions

It is possible, *without referring to any theoretical model,* to derive from our experimental data information concerning the shape of cell survival curves. We have to assume only that *an equal level of tissue reaction corresponds to an equal survival level for stem cells.* This hypothesis seems reasonable for the irradiation protocols compared [9].

In a dose–survival diagram (Fig. 4), the logarithm of the surviving fraction of cells (in arbitrary units) is plotted against dose (in rad). To obtain the two first points of such a survival

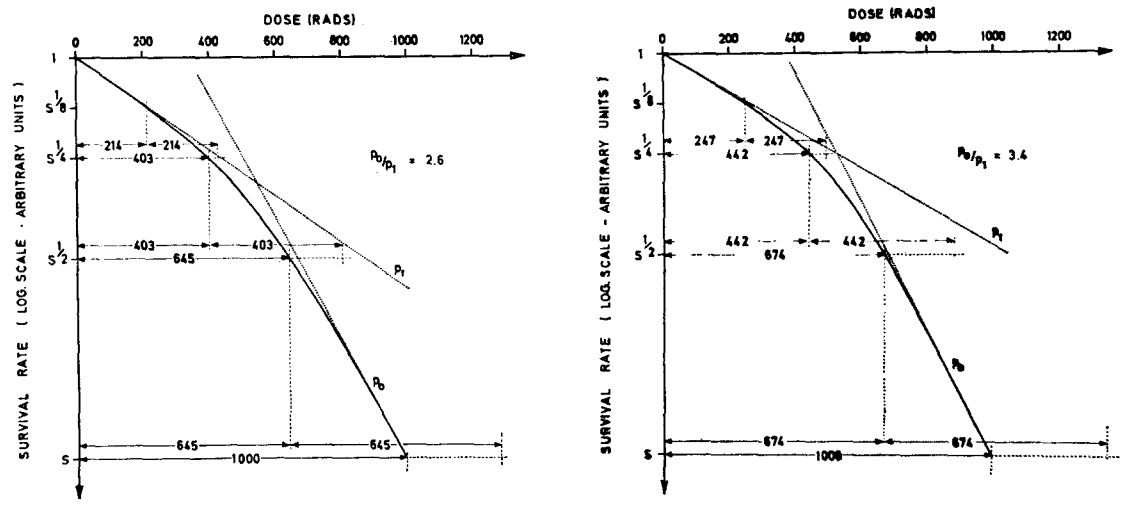


Fig. 4. Direct determination of the shape of cell survival curves from observation of gross tissue reactions. The slopes p_1 *at the origin and* p_0 *for relatively high doses (about* 1000 *rad) are compared. If one assumes a mean lethal dose of* 150 *rad for high doses, a ratio* $p_0/p_1 = 2{\cdot}6$ *(on the left) or* 3·4 *(on the right) corresponds to a mean lethal dose for "direct lethal events" of* 400 *or* 500 *rad respectively (see text).*

curve, we derive from Fig. 2 that the total dose for LD_{50} increases from 1000 to 1290 rad if a single dose D_s is split into two equal fractions ($i = 3{\cdot}5$ hr). Thus a single dose of 1000 rad and two doses of 645 rad produce the same effect, i.e. they correspond to the same survival level S. The second point of the survival curve is obtained assuming that each "subfraction" of 645 rad reduces survival rate in the same proportion, i.e. to $S^{\frac{1}{2}}$.

This reasoning can be extended, deducing again from Fig. 2 that 645 rad and 2×403 rad $= 806$ rad reduce the survival rate to the same extent $S^{\frac{1}{4}}$ and that 403 rad reduces the survival rate to $S^{\frac{1}{8}}$. In this way a "survival curve" is obtained directly from the observation of gross tissue reactions. It has no ordinate scale, but allows comparison of the slopes p_1 at the origin and the slope p_0 for high doses (about 1000 rad). The ratio of the slopes $p_0/p_1 = 2{\cdot}6$. This ratio of 2·6 is probably a *lower limit*. In particular, if for instance we assume that, for the considered interval i, only 80% of complete recovery was achieved (which seems to be an extreme assumption), we obtain a ratio of the slopes which is 3·4. Accepting for doses of about 1000 rad a reasonable value of 150 rad for the mean lethal dose ($p_0 = 1/150$), it can be calculated that the slope p_1 at the origin corresponds to a mean lethal dose of 400 ($p_0/p_1 = 2{\cdot}6$) or 500 rad ($p_0/p_1 = 3{\cdot}4$). These values are in agreement with the results obtained more than 10 years ago by Barendsen with cell cultures [10].

It seems reasonable to assume that the slope at the origin p_1 refers to "direct lethal events"; it corresponds to the "linear component" of the dose effect curve. The slope for higher doses p_0 can be ascribed to killing mechanisms which are the combination of direct lethal events and "accumulation of sublethal events". This latter corresponds to the "quadratic component" of the dose effect curve in terms of the "dual radiation action therapy" of Kellerer and Rossi [11].

C. *Implications for neutron therapy*

As far as RBE is concerned, it is well-known that RBE increases when the dose decreases [11]. However, for *cell killing*, a continuous increase of RBE when dose decreases is unlikely: the upper limit should be the ratio of the initial slopes for X-rays and neutrons. As far as a differential effect *related to fraction number* between different tissues is concerned, we have seen that such an effect is unlikely for X-rays, for small doses per fraction and probably also for higher doses. The same should be a fortiori true for neutrons, where direct lethal events are relatively more important than for X-rays.

However, this does not exclude the possibility of a differential effect between two tissues related to *radiation type:* for example the difference of the initial slope between X-rays and neutrons may be more important for one tissue than for the other. These conclusions have been derived from observations of *some* reactions of *some* tissues; their applicability to any other biological effect needs to be checked, particularly for long term effects.

REFERENCES

1. M. M. Elkind and H. Sutton, X-ray damage and recovery in mammalian cells in culture. *Nature (Lond.)* **184,** 1293 (1959).
2. J. Dutreix, A. Wambersie and C. Bounik, Cellular recovery in human skin reactions. Application to dose, fraction number, overall time relationship in radiotherapy. *Europ. J. Cancer* **9,** 159 (1973).
3. A. Wambersie, J. Dutriex and J. Gueulette, Determination of the shape of mammalian cell survival curves by the evaluation of cellular recovery as the function of the size of the fraction. Comparison with theoretical models. In *Proc. IVth Symp. on Microdosimetry*, Verbania-Pallanza, Italy, 1973. Comm. of the Eur. Communities, Brussels, (In press).
4. J. Dutreix, F. Eschwege. A. Wambersie and E. Salamon, Early recovery of skin reactions in mice after 15 sessions of split-dose irradiation. In *Proc. Conf. on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, Carmel, Calif., 1969 BNL Report 50203 (C-57), p. 286 (1970).
5. A. Wambersie, J. Dutriex and J. Lellouch, Cellular recovery as a function of dose per fraction. Experiments on mouse skin and intestine; preliminary results on man. In *Proc. Second Congress of the Eur. Ass. of Radiology*, Amsterdam, The Netherlands, 1971. Excerpta Medica, Amsterdam, p. 402 (1972).
6. H. R. Withers, Recovery in normal and malignant tissues. In *Proc. IVth Int. Congress of Radiation Research*, Evian, (1970) (In press).
7. J. Denekamp, Changes in the rate of repopulation during multifraction irradiation of mouse skin. *Brit. J. Radiol.* **46,** 381 (1973).
8. A. Wambersie and J. Dutreix, Effects of fractionation in radiotherapy. Repopulation of normal tissues during treatment. In *Proc. IVth Int. Congress of Radiation Research*, Evian, (1970) (In press).
9. H. R. Withers and M. M. Elkind, Radiosensitivity and fractionation response of crypt cells of mouse jejunum. *Radiat Res.* **38,** 598 (1969).
10. G. W. Barendsen, Dose–survival curves of human cells in tissue culture irradiated with alpha-, beta-, 20-kV X- and 200-kV X-radiation. *Nature, (Lond.)* **193,** 1153 (1962).
11. A. M. Kellerer and H. H. Rossi, The theory of dual radiation action. *Cur. Top. Radiat. Res. Quart.* **8,** 85 (1972).

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Repair and Repopulation in Mouse Skin During Fractionated Neutron and X-Irradiation

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Abstract—*The relative importance of repair and repopulation after single large doses and after multiple small doses has been investigated both for X-rays and neutrons, using a new experimental design. A considerable dose increment is necessary within* 24 *hr after* 1000 *rad of X-rays or* 450 *rad of neutrons; no dose increment is necessary in the following* 8 *days, but by* 15 *days* 400 *rad are necessary in both cases to counteract repopulation. During multifraction irradiation (with* 4, 9 *or* 14 *fractions of* 100 *rad of neutrons or* 300 *rad of X-rays) considerable dose sparing is observed. However,* 24 *hr after the last fraction of X-rays, a large dose increment was necessary, but after neutrons the skin was more sensitive (i.e. a negative increment); this is presumed to be a result of synchrony.*

The repopulation rate increased with increasing number of fractions with X-rays. After 4 *and* 9 *fractions of neutrons a similar result was obtained. But after* 14 *fractions of neutrons it appeared that the repopulation in* 8 *days was much smaller than after X-rays.*

RBE values of 2·2 *at* 1000 *rad of X-rays single dose and* 3·0 *at* 300 *rad of X-rays per fraction were confirmed.*

INTRODUCTION

GROSS SKIN reactions after irradiation probably reflect the survival of the basal layer cells of the epidermis as the $LD_{50/4d}$ reflects the survival of intestinal crypt cells. Experiments in which the survival of epidermal clones *in vivo* and gross skin reactions on the feet of mice have been compared give very similar responses in terms of the dose increments required to counteract repair and repopulation, and in terms of the RBE of fast neutrons relative to X-rays [1, 2]. Thus the dose response curves which have been measured by using skin reactions will be interpreted in terms of cell survival.

Some processes which are likely to occur in normal tissues between fractions are:

1. The repair of sublethal injury which for X-rays is complete within 8–24 hr [3].
2. Reassortment of cells throughout the cell cycle because of differences in the sensitivity of cells of different ages, and also because of the accumulation after a dose of radiation in particular parts of the cell cycle; for example accumulation in G_2 because of mitotic delay [1].
3. Repopulation of the survivors in response to the reduction in population size [1, 4].

We are interested in the relative contributions of these three processes in organised tissue during fractionated radiotherapy, both with X-rays and with neutrons. It is known that less sublethal injury can be accumulated after neutron irradiation than after X-irradiation, i.e. the extrapolation number is lower for neutron than for X-ray survival curves. Similarly

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the value of $(D_2 - D_1)_{24hr}$ measured in a number of normal tissues is smaller after neutrons than after X-rays [e.g. 2, 5, 6]. It has been found that $(D_2 - D_1)_{24hr}$ is large, after a single priming dose of X-radiation for skin, for gut, and for several other normal tissues when measured *in vivo* [1, 5–7]. If this dose increment is interpreted in terms of D_q, then it is considerably larger than most values found for D_q when measured for cells *in vitro*. Similar large values for D_q have however been measured for tumour cells grown as spheroids [8], but D_q was reduced if the cells were irradiated as a single cell suspension. Thus some intimate cell contact may be necessary, as generally occurs in organised tissue in order to achieve the large amount of recovery of sublethal injury associated with a large D_q.

The variation in sensitivity through the cell cycle and the consequent synchrony that is induced by a priming dose of irradiation can be considerable in skin after X-irradiation [1]. However, this variation in sensitivity may be smaller after high LET radiation than after a low LET radiation such as X-rays [9,10]. In this case less effects of synchrony would be expected with neutrons both *in vitro* and in tissues measured *in vivo*.

The contribution of repopulation to the dose increment required between fractionated doses is not well understood in any tissue. The timing of the response probably depends upon the time at which the death of cells which have been damaged by the irradiation is recognised by the organism. This will be dependent on both dose, the cell cycle time, (which will differ in different tissues) and possibly the turnover time of the differentiated component of the tissues, (which may differ in the same tissue measured in different parts of the body, e.g. as a function of skin thickness). It is possible that the time at which damage to the basal layer is perceived by the surviving cells may depend on the time over which the superficial layers are removed.

There is no *a priori* reason for differences in the rates of reaction after irradiation with X-rays or neutrons, if equivalent biological damage is being caused and is being registered in the same cells in the same overall time. Early attempts to investigate this were based on studies of the pattern of healing of the skin reaction after equivalent peak reactions, caused with X-rays or neutrons [11]. The rate of development of the reaction does not appear to be dose dependent but the peak and healing rate are, and are believed to depend on the level of depletion of the basal layer and the rate of repopulation [11]. For equivalent peak reactions the healing rates matched well after single or fractionated treatments [12].

The present paper will describe a new design of experiment aimed at measuring the relative contribution of repair and repopulation after fractionated irradiation performed with X-rays or neutrons. The extent of repair of sublethal injury will be measured by the dose increment required in the 24-hr interval after a pretreatment course. This will of course be interfered with by any synchrony induced in the population which might be expected to be greater after X-rays than after neutrons [9, 10]. The repopulation component will be estimated from the additional dose required if an interval greater than 24 hr, for example 8 days, is allowed between the pretreatment and the time of testing. In this case the dose increment between one and eight days will be interpreted as dose necessary to sterilise new cells born through the process of repopulation.

The skin reaction of the left hind foot of albino mice has been measured in all the experiments which will be described below. Two strains of mice have been used, SAS/TO non-inbred mice and WHT inbred albino mice. The foot is irradiated with some form of pretreatment, X-rays or neutrons, with various fractionation schedules followed by graded test doses of X-rays to give a dose response curve at various times later (Table 1).

Table 1

Pretreatment	Tested with a range of X-ray doses to obtain a measurable response at
1000 rad X-rays	0, 1, - - - - - 15 days
4 × 300 rad X-rays	0, 1, - - - - - 15 days
9 × 300 rad X-rays	0, 1, - - - - - 15 days
14 × 300 rad X-rays	0, 1, - - - - - 15 days
450 rad neutrons	0, 1, - - - - - 15 days
4 × 100 rad neutrons	0, 1, - - - - - 8 days
9 × 100 rad neutrons	0, 1, - - - - - 8 days
14 × 100 rad neutrons	0, 1, - - - - - 8 days

After irradiation the skin reactions on the feet of the animals have been scored on a scale of erythema through dry desquamation to ulceration, from shortly after irradiation either 3 or 5 times per week for at least 30 days. The skin scoring system has been described in detail in many previous publications [e.g. 3, 4]. Dose–response curves have been constructed by taking the average skin reaction over a period of 8–30 days after single doses or over an equivalent period for the fractionated doses,

determined by matching the leading edges of the reaction vs time curves for a fairly high reaction level.

RESULTS

The dose–response curves obtained using X-rays only are shown in Fig. 1. The average

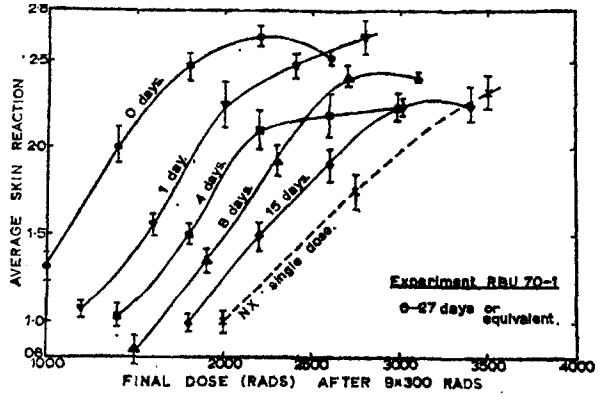


Fig. 1. The average skin reaction vs final test dose for mice given 9 daily fractions of 300 rad of X-rays, followed by test doses at the interval shown against each curve. A single dose is included for comparison.

reaction is shown as a point for each group of animals and curves have been constructed through those points for animals which were given the same fractionation schedule. A curve is also shown for animals which have been given a single dose of X-rays, i.e. no pre-treatment. The first curve from the left is for animals which have been given 9 daily fractions of X-rays followed by graded test doses immediately after the last 300 rad fraction. This is labelled "0 days". The curve labelled "1 day" is for animals which were given their graded test doses of X-rays 24 hr after the last of the 9 small fractions. Similarly curves are shown for animals which were given their range of test doses at 4, 8 or 15 days after the last of the nine 300 rad X-ray fractions.

It can be derived e.g. that for a skin reaction of 1·5 a total dose of 3800 rad is required (i.e. $9 \times 300 + 1100$) if the test dose is given immediately after the ninth fraction, versus a dose of 2500 rad for a single dose of X-rays. This is probably because of the processes of repair, synchrony, and repopulation occurring between each of the 9 fractions, leading to additional dose being necessary. By comparing the single dose curve with the 0 day curve an average dose increment for each of the 8 intervals can be calculated and this is equivalent to approximately 200 rad per fraction interval. If we then compare the 0 day curve with the 1 day curve we obtain the dose increment which we interpret as being necessary to counteract repair after the last of the nine fractions, repair after the first eight being assumed to be complete. This dose is considerable, and is in excess of 300 rad after the last 300 rad dose. The separation of the 1 day curve, and the curves obtained when a greater time is allowed to elapse before giving the test doses, gives us an indication of the dose increment necessary to counteract repopulation after the repair of sublethal injury following 9 daily fractions of 300 rad. In this particular experiment the curves are well separated showing that additional dose is necessary to counteract repopulation in a situation where the cells of the epidermis have been induced to proliferate more rapidly because of the damage caused by the nine fractions of X-rays. This has been discussed in detail elsewhere [4].

Figure 2 shows a similar experiment in which the test doses of X-rays have been given after 9 fractions of 100 rad of neutrons. Again the average dose required to counteract repair of sublethal injury can be derived from the relative positions of the single dose and the "0 day" curves. The curve obtained 24 hr after the last of the nine fractions is displaced to the left of the curve obtained if the test doses are given at day 0. This indicates that there is a negative dose increment necessary 24 hr after the last fraction, i.e. that the population is more sensitive 24 hr after the last fraction than immediately after the last fraction. The curve for animals tested at 8 days after the last of the 9 neutron fractions is displaced well to the right of the 0 and 1 day curves, indicating that this additional dose is necessary

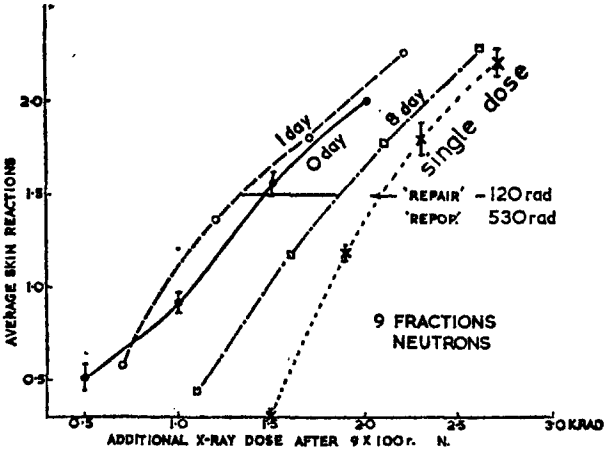


Fig. 2. The average skin reaction vs final test dose for mice given 9 daily fractions of 100 rad of neutrons at the interval shown against each curve. A single dose is included for comparison.

to counteract repopulation after treatment with 9 fractions of neutrons.

DISCUSSION

One way of comparing the results which have been obtained with neutrons and with X-rays is shown in Fig. 3. Cuts have been taken across the curves in Fig. 1 and Fig. 2 at a reaction level of 1·5. In Fig. 3 the *additional dose of X-rays* which is necessary to produce this reaction level after a single, large priming dose, or after 4, 9 or 14 small fractions is shown plotted against the time interval after

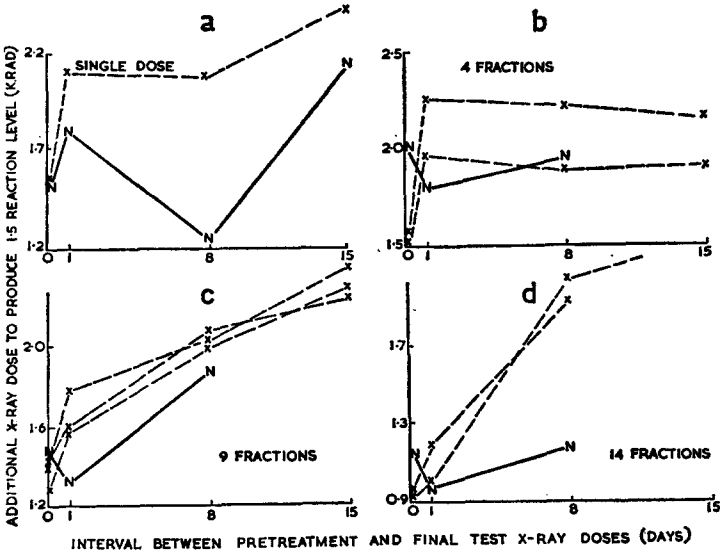


Fig. 3. The additional X-ray dose necessary to produce an average reaction level of 1·5 *against the interval between pretreatment and the final test X-ray doses. (a) After* 1,000 *rad of X-rays or* 450 *rad of neutrons; (b) after* 4 × 300 *rad of X-rays or* 4 × 100 *rad of neutrons; (c) after* 9 × 300 *rad of X-rays or* 9 × 100 *rad of neutrons; (d) after* 14 × 300 *rad of X-rays or* 14 × 100 *rad of neutrons. For (b), (c) and (d): different lines refer to results of different experiments.*

priming dose of 450 rad of neutrons the dose increment necessary in the first 24 hr is approximately 275 rad of X-rays; between day 1 and day 8 no dose increment is necessary (negative), suggesting that synchrony is obscuring any effect due to repopulation. However, the dose increment necessary between day 1 and day 15 is approximately 450 rad of X-rays. As after the nine X-ray fractions this is equivalent to approximately 30 rad per day necessary to counteract repopulation over this whole period, but the effect is not apparent for the first 8 days.

Figure 3(b) shows the effect after 4 fractions of X-rays or neutrons. After 4 fractions of the last fraction at which the range of test X-ray doses was given. Thus, in Fig. 3(a) the additional X-ray dose is plotted against days after giving a single dose of 1000 rad of X-rays or 450 rad neutron (chosen using an RBE value from Field and Hornsey [13]). Points are shown at 0 days, i.e. immediately after the test dose, at 1 day, 8 days and 15 days. From these curves the amount of dose necessary to counteract repair of sublethal injury, together with the induced synchrony, and the dose necessary to counteract repopulation can be seen. After 1000 rad of X-rays approximately 550 rad are necessary to counteract repair in the first 24 hr. Thereafter approximately 30 rad per day are necessary to counteract repopulation over the next two weeks. After a X-rays additional dose is necessary in the first 24 hr to counteract the repair of sublethal injury; in the 8 or 15 days following this however no dose is necessary to counteract repopulation. After 4 fractions of neutrons no additional dose is necessary in the first 24 hr. The cells appear to be more sensitive 24 hr after the 4th fraction than immediately after the 4th fraction, by about 300 rad. Between 1 and 8 days a very small additional dose is necessary which however is still not as great as the dose needed immediately after the fourth fraction.

In Fig. 3(c) the results of the test radiation after 9 fractions of X-rays or neutrons are shown. After 9 fractions of X-rays approximately 300 rad were necessary in the following

24 hr to counteract repair. Between 1 and 8 days approximately 60 rad per day are necessary to counteract repopulation, but beyond 8 days a lower value of about 20 rad per day is required. After 9 fractions of neutrons, however, a negative increment appeared to be necessary to counteract repair in the first 24 hr, equal to about 150 rad of X-rays. Beyond 24 hr a positive dose increment is necessary again between 1 and 8 days in order to counteract repopulation and this amounts to almost 80 rad of X-rays per day. This is very similar to the value observed after 9 fractions of X-rays, as shown by the similarity of the slopes in Fig. 3(b).

In Fig. 3(d) the response after 14 fractions of X-rays or neutrons are shown. After 14 fractions of X-rays approximately 200 rad are required in the next 24 hr to counteract repair, whereas the dose required to counteract repopulation in the following week has increased enormously to between 100 and 150 rad/per day. This is equivalent to a cell doubling time of less than one day. After 14 fractions of neutrons however a negative increment was again observed for the first 24 hr of about 150 rad. Beyond 24 hr, between the 1st and 8th day, a very small additional dose is necessary to counteract repopulation being only approximately 30 rad per day, i.e. quite different from the measurements obtained after 14 fractions of X-rays.

These results are summarised in Fig. 4(a) and (b). Fig. 4(a) shows the "repair" occurring in 24 hr (measured as the dose increment

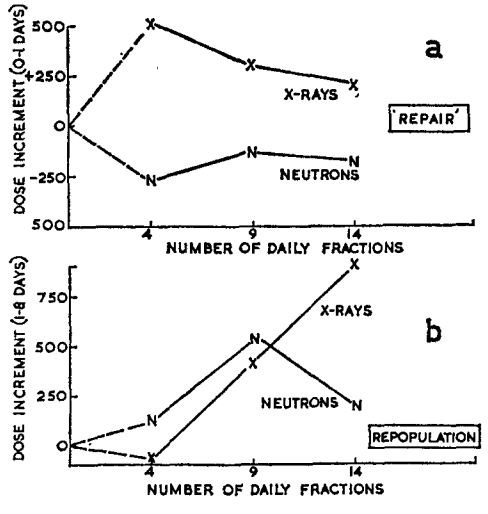


Fig. 4. The dose increments necessary to counteract "repair of sublethal injury" and "repopulation" as a function of the number of daily fractions (of 100 rad of neutrons or 300 rad of X-rays). (a) The dose increment in the first 24 hr following the last daily fraction. This is positive after X-rays but negative after neutrons; (b) The dose increment needed between 1 and 8 days after irradiation. This is similar after X-rays and neutrons except for the 14 fraction experiments.

Table 2. X-ray dose increments necessary to obtain an average skin reaction of 1·5 after pretreatment.

	Pretreatment			
	1F	4F	9F	14F
X-ray dose at day 0:				
X	1550	1530	1300	940
N	1500	2030	1460	1130
Day 1–day 0 "repair":				
X	525	520	300	210
N	270	−260	−150	−170
Day 8–day 1 "repopulation":				
X	−10	−70	400	920
N	−300	130	540	200
Day 15–day 1 "repopulation":				
X	400	−30	560	1040
N	400			

necessary in the first 24 hr after the last fraction in order to produce the standard reaction level of 1·5), against the number of fractions used as pretreatment. The dose increment is positive for all the multifraction X-ray treatments, but negative after neutrons. In Fig. 4(b) the increment necessary to counteract repopulation between day 1 and day 8 is shown against the number of fractions. The response after 4 fractions and after 9 fractions is very similar, but not after 14 fractions. This data is also shown in Table 2 where the dose increments necessary after the various pretreatments are shown.

CONCLUSIONS

1. The RBE predicted from Field's curve [13] of RBE as a function of dose per fraction has fairly accurately predicted the neutron dose that was equivalent to 1000 rad of X-rays (450 rad of neutrons, RBE = 2·2) and the dose equivalent to 300 rad fractions of X-rays (100 rad of neutrons, RBE = 3·0). Figures 3(b), (c) and (d) show that slightly more X-ray dose was necessary immediately after 4, 9 or 14 fractions of neutrons than after the equivalent X-ray scheme. However, if the doses administered

one day later are considered, when repair from the last fraction has been allowed, somewhat smaller additional doses are necessary after neutrons than after X-rays. Thus an average of about 3 is obtained, being a little larger or smaller depending on which criterion is used.

2. There was a considerable dose increment necessary in the 24 hr following 4, 9 or 14 fractions of X-rays; in each case with a similar neutron schedule a negative increment was required, i.e. the skin was more sensitive 24 hr after than immediately after the last fraction. This result is not understood. It seems likely that it is attributable to synchrony of the surviving cells, which are in a sensitive phase 24 hr after the fourth, ninth or fourteenth fraction of neutrons, but in a more resistant phase after an equivalent schedule of X-rays. We did not anticipate such an effect of synchrony after neutron irradiation.

3. The average dose increment required to counteract the processes occurring between the 4, 9 or 14 fractions can be estimated by comparing the single dose required to give a 1·5 reaction level (2080 rad X-rays) with the additional dose required 0 days after the last fraction (Table 2). Only 50 rad less of X-rays is necessary after 4 fractions of neutrons, 620 less after 9 fractions and 950 less after 14 fractions. Thus a large part of each neutron fraction is "repaired" when the average values are considered.

4. The dose necessary to counteract repopulation in the 8 days following the last of the fractionated treatments is very similar after 4 or 9 fractions of neutrons or X-rays; the results are probably within the experimental errors of the system. However, after 14 fractions of neutrons, the very large increase in the rate of repopulation which was observed after 14 fractions of X-rays is no longer evident. The reason for this is not understood. It is the first time that a suggestion of reduced proliferation rates after neutrons has been obtained experimentally and does not agree with the similar rates of healing observed after fifteen equal fractions in other experiments. Further experiments are clearly needed to clarify the mechanisms involved.

REFERENCES

1. 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,**36 1 (1969).
2. J. Denekamp, E. W. Emery and S. B. Field, Response of mouse epidermal cells to single and divided doses of fast neutrons. *Radiat. Res.* **45,** 80 (1971).
3. J. F. Fowler, K. Kragt, R. E. Ellis, P. J. Lindop and R. J. Berry, The effect of divided doses of 15 MeV electrons on the skin response of mice. *Int. J. Radiat. Biol.* **9,** 241 (1965).
4. J. Denekamp, Changes in the rate of repopulation during multifraction irradiation of mouse skin. *Brit. J. Radiol.* **46,** 381 (1973).
5. D. K. Bewley, J. F. Fowler, R. L. Morgan, J. A. Silvester, B. A. Turner and R. H. Thomlinson, Pre-therapeutic experiments with the fast neutron beam from the MRC Cyclotron. VII. Experiments on the skin of pigs with fast neutrons and 8 MV X-rays including some effects of dose fractionation. *Brit. J. Radiol.* **36,** 107 (1963).
6. S. Hornsey, The effectiveness of fast neutrons compared with low LET radiation on cell survival measured in the mouse jejunum. *Radiat. Res.* **55,** 58 (1973).
7. T. L. Phillips, Discussion in *Proc. Conf. on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, Carme, Calif., 1969. BNL Report 50203 (C-57), p. 194 (1970).
8. R. E. Durand and R. M. Sutherland, Effects of intercellular contact on repair of radiation damage *Exp. Cell Res.* **71,** 75 (1972).
9. E. J. Hall, W. Gross, R. F. Dvorak, A. M. Kellerer and H. H. Rossi, Survival curves and age response functions for Chinese hamster cells exposed to X-rays or high LET alpha particles. *Radiat. Res.* **52,** 88 (1972).
10. R. Bird and J. Burki, Inactivation of mammalian cells at different stages of the cell cycle as a function of radiation linear energy transfer. In *Biophysical Aspects of Radiation Quality*, IAEA, Vienna, p. 241 (1971).
11. 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. *Radiat. Res.* **29,** 71 (1966).

12. 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).
13. 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 (1967).

Europ. J. Cancer Vol. 10, pp. 249–252. Pergamon Press 1974. Printed in Great Britain

Response of Jejunal Mucosa to Fractionated Doses of Neutrons or γ-Rays*

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Abstract—*The $RBE_{n+\gamma/\gamma}$ of fast neutrons produced by the $^{9}Be(d,n)^{10}B$ reaction ($E_d = 50$ MeV) relative to ^{60}Co γ-rays for killing of jejunal crypt stem cells has been measured over a wide range of doses beginning at 400 rad of γ-rays, and extending to doses as high as 1600 rad. Dose-equivalent values for recovery as a function of the size of the dose-per-fraction have been measured. When γ-ray doses up to about 400 rad are given, about 60% of the dose is "recovered". With neutron doses of 100–200 rad, the recovered dose amounts to only about 13% of the given dose.*

INTRODUCTION

THE RELATIVE biological efficiency of neutrons relative to γ-rays [$RBE_{(n/\gamma)}$] that is relevant to radiotherapy is the value at the dose to be used for each dose-fraction. In preclinical experimental studies of tissue response, the levels of injury that can be evaluated accurately are usually only achieved by single doses that are large relative to the dose-per-fraction used by radiotherapists. Hence, single dose experiments are of limited value and it is necessary that animal tissue responses to multifraction irradiation be evaluated using doses-per-fraction of the same size as are likely to be used for treating patients. This paper presents some radiobiology of the mouse jejunal mucosa obtained in the course of preclinical measurements of $RBE_{(n/\gamma)}$ values using multifraction irradiation.

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MATERIAL AND METHODS

1. *Response assay*

The survival response of mouse jejunal crypt stem cells was assayed by an *in situ* cloning technique described elsewhere [1]. Briefly, animals were given total-body exposures of either γ-rays or neutrons. Three and one half days later, animals were sacrificed and transverse sections of the jejunum were prepared for histology. The number of crypts per circumference was counted under the microscope and plotted as a function of dose on semi-logarithmic co-ordinates. Survival curves were fitted to the data using a least-squares regression analysis. Data are presented with their 95% confidence limits.

2. *Mice*

Female C_3Hf/Bu mice from a specific-pathogen-free colony maintained in our laboratory, were irradiated when 8–10 weeks of age.

3. *Irradiation*

Neutrons were generated at the Texas A and M University variable energy, sector-

focussed 88-in cyclotron (TAMVEC) by bombarding a beryllium target with a beam of 50 MeV deuterons ($E_d = 50$ MeV (Be)). The dose in rad was computed using the Bragg–Gray relationship and methods discussed in an earlier paper [2]. Based on this previous work, the entrance skin dose is about 65% of the maximum dose which occurs just forward of mouse midline and is attenuated by about 1% in penetrating the latter half thickness of the mouse. The gamma contribution to total dose is about 5% [2]. Doses are expressed as the total neutron + γ-ray dose.

The dose rate used was 80–90 rad/min. Exposures were monitored by two back-to-back sealed transmission chambers connected to integrators and placed in the neutron beam. When the pre-determined charge for the required dose was accumulated, the deuteron beam was automatically blocked.

The mice were positioned individually in 8 plastic holders within a 15 cm dia circle on the front face of a 40 cm cubical lucite tank containing tissue-equivalent liquid [3]. The radiation field size was 39×39 cm and the mice were centered in the field at a distance of 140 cm from the target. Over the 15 cm circle which contained the mice, the measured midline dose was uniform to within $\pm 5\%$. Midline doses in rad were determined by use of a 0·1 cm^3 tissue equivalent ion chamber enclosed within a $1{\cdot}9 \times 1{\cdot}9 \times 7$ cm block of Shonka A–150 T.E. plastic [4], which was used as a mouse phantom and inserted in one of the holders.

A ^{60}Co radiotherapy machine was used for γ-ray experiments. Mice were exposed 5–10 at a time in a well ventilated lucite box at a source to mid-mouse distance of 101·5 cm. Exposure rate was checked before each experiment using a Victoreen ionization chamber. The dose rate varied slightly throughout the series of experiments but was approximately 100 rad/min.

When the dose was given as multiple fractions, the interval between fractions was 3 hr for both neutrons and γ-rays. This was considered sufficient time for essentially complete repair of sublethal injury and yet was short enough to minimize the extent of regeneration of surviving cells during the latter part of the more protracted fractionation regimes.

RESULTS

Survival curves from single dose and multiple equal dose fraction experiments using neutrons and ^{60}Co γ-rays are shown in Fig. 1. For clarity, a survival curve for 20 equal γ-ray doses is excluded because it ranges between 2800 and 4200 rad. Also for clarity, only the best-fitting curves are shown for the multifraction neutron data. The four most obvious aspects of the data in Fig. 1 are:

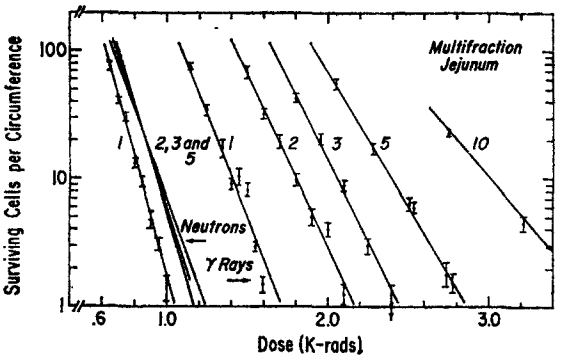


Fig. 1. Single and multifraction dose-survival curves for crypt stem cells of mouse jejunum exposed to fast neutrons from the ^{9}Be(d, n)^{10}B reaction (E_d = 50 *MeV*) or to ^{60}*Co γ-rays. The numbers on each curve indicate the number of dose-fractions into which the total dose (shown on abscissa) was divided. Fractionation intervals were* 3 *hr. The multifraction neutron curves are superimposed: the multifraction γ-ray curves are displaced to higher doses for higher fraction numbers. The ranges shown are* 95% *confidence limits.*

1. There is an appreciable difference in the biological effectiveness of neutrons and γ-rays.
2. The multifraction dose–response curves are displaced towards higher doses, indicating that recovery occurs during fractionation intervals.
3. With increasing fractionation of dose, up to 10 fractions, there is a progressive shift of the γ-ray dose–survival curves to higher doses for the same range of cellular survival.
4. With neutrons, the 2, 3 and 5 fraction dose–survival curves are superimposed upon one another. Thus, the recovery per fractionation interval is obviously less in the larger fraction number regimes, (in which smaller doses-per-fraction were given).

Recovery occurring during each fractionation interval may be expressed in terms of recovered dose, $D_N - D_1/N-1$, where D_N and D_1 are the doses in N fractions and 1 fraction required to produce the same effect. Recovered dose is a function of the dose given and so, in Fig. 2, values for $D_N - D_1/N-1$, obtained from the data in Fig. 1, at a survival level of 10 cells/circumference, are plotted against the size of dose-per-fraction for both neutrons and γ-rays. Also shown in Fig. 2 are $D_N - D_1/N-1$ values obtained from multifraction experiments using E_d=16 MeV (Be) neutrons. The striking difference between the curves for recovery

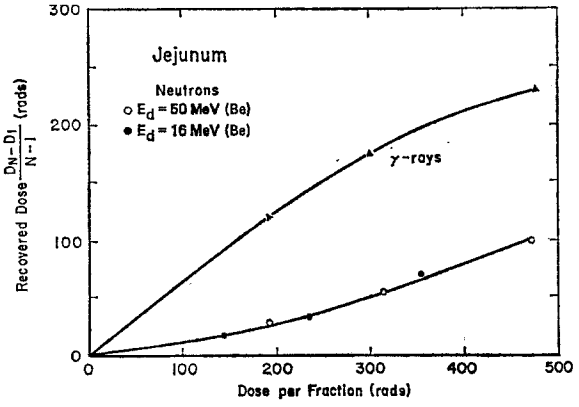


Fig. 2. Recovered dose is plotted as a function of given dose. With γ-rays, about 60% of the dose is recovered, although this value decreases at higher doses. With neutrons [either E_d = 16 MeV (Be) or E_d = 50 MeV (Be)] the recovery is less: between 100 and 200 rad, about 12–14% of the dose is recovered, but this increases to about 20% at doses of about 400 rad.

Table 1. $RBE_{n+\gamma/\gamma}$ values for E_d=50 MeV (Be) neutrons vs ^{60}Co γ-rays for killing of mouse jejunal crypt stem cells

γ-ray dose (rad)	400	425	450	500	550
$RBE_{n+\gamma/\gamma}$	2·8	2·7	2·61	2·45	2·34

from neutrons and gamma-rays is that the γ-ray curve is convex upwards whereas that for neutrons is concave. Thus, the proportion of the γ-ray dose recovered decreases with increasing dose whereas with neutrons, the proportion is still increasing at doses approaching 500 rad.

Values for $RBE_{(n/\gamma)}$, or more accurately $RBE_{(n+\gamma/\gamma)}$, for E_d = 50 MeV (Be) neutrons and ^{60}Co γ-rays may be determined directly from Fig. 1. Thus, for example, single doses of 1400 rad of γ-rays, and 840 rad of neutrons, both result in survival of 10 cells per circumference. Therefore, the RBE at this level of effect is 1·66. Values for RBE more useful to radiotherapists can be obtained from the multifraction curves. Five fractions of γ-radiation, each of 400 rad, results in the same cellular survival as does 5 doses of 143 rad of neutrons. Thus, the $RBE_{(n+\gamma/\gamma)}$ at the level of injury produced in jejunal crypts by 400 rad of ^{60}Co γ-radiation is 2·8. Similar measurements for RBE may be made for a wide range of doses from the data in Fig. 1. However, since experiments using more than 5 fractions were not carried out with neutrons, and since 400 rad per fraction was the lowest dose of γ-radiation given 5 times, that would reduce cell survival sufficiently to result in a readily-counted number of colonies, the RBE cannot be determined directly from Fig. 1 for doses lower than 400 rad of γ-rays. Values for $RBE_{(n+\gamma/\gamma)}$ for a range of doses that are in the range of potential clinical relevance are shown in Table 1.

DISCUSSION

Biological efficiency of neutrons relative to γ-rays

In planning the clinical use of neutrons, the aim is to achieve the same effect in normal tissues as is accepted from conventional X- or γ-radiation. Since there are logistic reasons for treating with fewer doses of neutrons than is conventional with X- or γ-rays, two factors must be accounted for: the $RBE_{n/\gamma}$, and the change in fractionation scheme. For example, if 5 doses of 200 rad of γ-radiation are usually given per week and this regime is to be replaced by 2 doses of neutrons per week, the RBE must be applied to the dose of γ-rays that would be given twice per week to produce the same effect as 5 fractions of 200 rad. When 5 daily doses of 200 rads/week of γ-radiation is replaced by 2 fractions of γ-radiation per week, the dose-per-fraction that produces equivalent acute responses (e.g. of the oropharyngeal mucosa) lies between 400 and 450 rad [5]. Thus, on the basis of the survival curves in Fig. 1 and the RBE values derived therefrom (Table 1), the appropriate dose of neutrons should lie between 145 and 175 rad, if the RBE for jejunal crypt stem cells is appropriate for the cells of other tissues involved in acute responses.

There is a considerable body of knowledge suggesting that the $RBE_{n/x}$ varies from tissue to tissue [6], and that values for gut injury are higher than those for other tissues. Our estimates match well with estimates made using *in vitro* systems such as Chinese hamster cells and bean roots exposed to E_d=50 MeV (Be) neutrons [7]. The RBE values shown in Table 1 are lower than those measured for intestine at the Hammersmith cyclotron, and are even lower than such estimates for skin damage [6]. However, the usefulness of such comparisons is limited, not only because of the difference in the energy spectrum of the neutrons (and for that matter of the X- and γ-rays), but also because of differences in measurement and expression of doses at Hammersmith and TAMVEC.

A general impression among radiotherapists is that fibrosis and other late effects of X- or γ-ray therapy are more severe when 5 fractions per week are replaced by 2, even though equal acute responses may be observed. Therefore, a dissociation between early and late responses

to twice weekly neutron treatments may be found, reflecting merely the change in fractionation pattern and not a characteristic of neutron-induced injury.

Recovery per frationation interval

It may be seen from Fig. 2 that when γ-ray dose–fractions of about 200 rad are given, approximately 60% of the dose is recovered, whereas only about 12–14% is recovered from a clinically-used dose fraction of neutrons [e.g. 125–175 rad $(n+\gamma)$]. It may be calculated from these data that the recovery of injury from exposure to the neutron beam is not merely recovery from the γ-ray-induced component of the injury. Thus, if all the recovery noted in Figs. 1 and 2 were due to repair of γ-ray injury, the γ-ray dose would have to represent $100/60 \times (12–14\%)$, or approximately 20% of the total measured dose. Since the γ-component is less than this, (about 5%), there must be some repair of neutron damage.

As the dose-per-fraction of neutrons increases, there is proportionately more recovery, $(D_N - D_1/N-1)$, as shown by the concavity of the neutron curve in Fig. 2. This may reflect an increase in "multi-event" (and, therefore, potentially repairable) cell-killing by both neutrons and the γ-ray component of the beam as the dose is increased. It may also reflect some influence of varying sensitivities of cells distributed throughout various phases of the division cycle.

Regardless of the radiobiological basis for the concavity of the curve for recovery from neutrons (Fig, 2), it is apparent that in the dose-range 125–200 $(n+\gamma)$ rad, the proportion of injury repaired changes slightly (from 12% to about 14%). Thus, giving the same total dose in a larger number of smaller dose fractions may not provide the additional sparing found with conventional γ-radiation, but rather, may result in a slightly greater biological effect.

REFERENCES

1. 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).
2. P. R. Almond, J. B. Smathers, G. D. Oliver, E. B. Hranitsky and K. Routt, Dosimetric properties of neutron beams produced by 16–60 MeV deuterons on beryllium. *Radiat. Res.* **54,** 24–34 (1973).
3. N. A. Frigerio, R. F. Coley and M. J. Sampson, Depth dose determinations I. Tissue equivalent liquids for standard man and muscle. *Phys. in Med. Biol.* **17,** 792–802 (1972).
4. F. R. Shonka, J. E. Rose, G. Failla, Second U.N. Conference on Peaceful Uses of Atomic Energy. Paper 753 (1958).
5. G. H. Fletcher and D. H. Hussey, (Personal communication).
6. 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).
7. E. J. Hall, J. K. Novak and S. A. Marino, Comparative radio-biological measurements with high-energy cyclotron-produced neutrons for radiotherapy. *Brit. J. Radiol.* (Submitted).

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Characteristics of Tumour Responses to Different Radiations and the Relative Biological Effectiveness of Fast Neutrons

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Abstract—*Responses of tumours to irradiation depend on the intrinsic radiosensitivity of the malignant cells, the oxygenation status of the cells and their kinetics of reoxygenation, variation of the sensitivity in the cell cycle, proliferation of cells in intervals of fractionated treatments and after treatment, and on the presence of non-proliferating cells. The influence of these parameters on the relative biological effectiveness of fast neutrons is discussed.*

INTRODUCTION

In the first decade after the development of techniques for the measurement of cellular clonogenic capacity, similarities between dose–survival curves of different types of mammalian cells have been emphasized to a greater extent than the differences in radiosensitivity which were observed as well. As more data became available, however, evidence was obtained that important differences exist among survival curves with respect to their shape in the shoulder region at low doses, as well as with respect to the slope of the exponential region at large doses. For X-rays, D_0 values range between 70 and 200 rad and extrapolation numbers between 1 and 100 have been observed. If such differences are also present for cells of human tumours, then a significant contribution would be expected to the differences in therapeutic margins obtained for different types of tumours in locations involving the same type of dose-limiting normal tissue.

In addition to the intrinsic radiosensitivity of the cells, tumour responses to fractionated irradiation are determined by the oxygenation status of the cells, their kinetics of reoxygenation, differences in sensitivity as a function of age in the cell cycle, proliferation of cells in intervals and after treatment and by the presence of non-proliferating cells. The purpose of this contribution is to evaluate the relative influence of these factors on the relative biological effectiveness (RBE) of fast neutrons for effects on tumours.

Fast neutrons will only provide a therapeutic advantage over X-rays or gamma rays, if the RBE for damage to the tumour is larger than the RBE for damage to those normal tissues which are dose-limiting in the treatment considered. Thus for the same location in normal tissue, tumours for which the largest RBE values are observed, would offer the best possibility to obtain a radiotherapeutic advantage from the use of fast neutrons.

DIFFERENCES IN INTRINSIC RADIOSENSITIVITY AMONG VARIOUS TYPES OF MAMMALIAN CELLS

In Fig. 1, a number of survival curves are presented, obtained with 300 kV X-rays and 15 MeV neutrons for cells from different normal tissues and tumours, investigated in the Radiobiological Institute [1]. These curves

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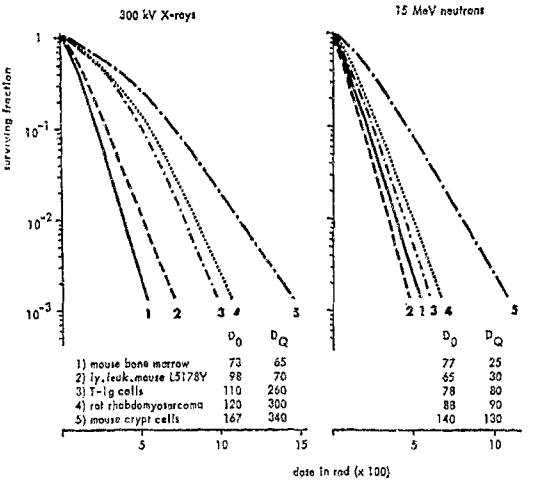


Fig. 1. Survival curves of different types of mammalian cells irradiated with 300 kV X-rays or 15 MeV neutrons. Curves 1: mouse haemopoietic stem cells; curves 2: mouse lymphocytic leukaemia cells L5178Y; curves 3: cultured T1g cells of human kidney origin; curves 4: rat R-1 rhabdomyosarcoma cells in culture; curves 5: mouse intestinal crypt stem cells.

illustrate that especially with X-rays a considerable variation in values of D_0 and D_Q is observed. With fast neutrons a reduction in shoulder width is generally obtained in comparison with X-rays, i.e. D_Q/D_0 ratios are smaller by factors ranging from 1·5 to 2·4. The D_0 values derived from the regions below 10% survival are in general also smaller for neutrons than for X-rays but these differences, expressed as $(D_0)_X/(D_0)_n$, range from about 1·0 to 1·5, i.e. the extent of the reduction is smaller than for the shoulder width. This indicates that the differences between fast neutrons and X-rays are more pronounced with respect to accumulation of damage than with respect to the sensitivity at large doses. It is further of interest to note that among the curves of Fig. 1, specific differences between cells from tumours and cells from normal tissues with respect to shapes of survival curves are not observed.

The first survival curve for mammalian cells obtained with high-LET alpha-radiation in 1959, demonstrated already that the RBE is not constant, but depends on the level of damage or the dose considered [2, 3]. Comparison of the RBE values derived from the curves in Fig. 1, tabulated in Table 1, shows that in general at larger doses or lower fractions of surviving cells the RBE values are smaller than at small doses or high survival. In addition, a considerable spread in RBE values is observed by a factor of about 2 among different types of cells. The differences in RBE values illustrated for 15 MeV neutrons may be augmented for fast neutrons of lower energies for which generally larger RBE values are obtained. For radiotherapeutic applications the comparison of RBE values at a fixed dose level is of greater interest than RBE values at a specified level of survival. In these applications the dose per fraction and the fractionation schedule will be determined to a large extent by the tolerance of the normal tissues which, at least for X-rays, generally show a smaller variation among patients than the tumour sensitivity. RBE values for fixed dose levels are also given in Table 1.

With respect to the influence of repair of sublethal damage it is important to note that D_Q/D_0 ratios provide only a measure of the relative width of the shoulder of a survival curve, but the precise shapes of the curves at low doses between 100% and 10% survival are frequently inaccurately known, especially with regard to the magnitude of the linear component [3, 4]. Because in radiotherapy doses are commonly administered in daily fractions of 100–400 rad of X-rays or gamma rays, RBE values of fast neutrons for this region are the most important and they should be determined with better accuracy than the data presently available for most experimental tumours and normal tissues.

It may be concluded that if survival curves of cells in human tumours show a similar spread in RBE values as measured for the systems represented in Fig. 1, then large variations in RBE values by a factor of 2 must be expected, due to differences in intrinsic radiosensitivities of the cells to X-rays and neutrons. This would imply that for some types of tumours fast neutrons might not provide a therapeutic gain over X-rays at all, while for other types of tumours the gain might be considerable. The variation in RBE values by a factor of 2 shown in Table 1 might represent a maximum, but the variation in RBE values by a factor of 1·3 obtained if the data for cells originating from haemopoietic tissues are excluded, still indicates that very important differences exist. It will be a main object of clinical studies to determine the types of tumours for which a useful gain can be obtained. The data of Table 1 and other data in the literature suggest that for cells from tumours originating from the haematopoietic and lymphatic tissues relatively low RBE values are observed. If this applies similarly to human tumours, it would imply that for these types of tumours fast neutrons are less likely to provide a therapeutic advantage over X-rays than for tumours originating from other tissues.

Table 1. RBE values derived from survival curves of different types of cells

	At 50% survival	At 10% survival	At 100 rad of 15 MeV neutrons	At 200 rad of 15 MeV neutrons
Mouse haemopoietic stem cells	1·5	1·1	1·3	1·1
Mouse lymphocytic leukaemia cells L5178Y	2·1	1·6	1·8	1·6
Cultured cells of human kidney origin	2·7	2·0	2·5	2·2
Rat rhabdomyosarcoma cells	2·6	2·0	2·5	2·2
Mouse intestinal crypt stem cells	1·8	1·6	1·9	1·8

ANOXIC CELLS AND REOXYGENATION IN TUMOURS

The presence of anoxic or severely hypoxic cells in tumours and the observation that the oxygen enhancement ratio (OER) of fast neutrons is smaller by a factor of about 1·6 than the OER of X-rays, has long been considered to constitute the principal rationale for the use of fast neutrons in radiotherapy [5, 6]. The ratio $(OER)_x/(OER)_n$ has been called the gain factor, but it is evident that this ratio represents only the maximum gain factor for the oxygen effect, which would be obtained if all cells in a tumour were anoxic and remained anoxic throughout a treatment course [6–9]. The effective gain factor due to the oxygen effect will in general be smaller than the maximum because not all cells in a tumour are anoxic and because in fractionated treatments reoxygenation may occur. This reoxygenation was first documented by Van Putten and Kallman [7] for a C3H mouse sarcoma and by Thomlinson for a sarcoma RIB5 in rats [8]. Studies of the time course of reoxygenation have shown that considerable differences exist among types of tumours [8]. Consequently, in order to eliminate the influence of anoxic cells as much as possible, tumours would require individually optimalized fractionation regimens. However, no ways are presently available to devise such tailor-made schemes for human tumours. The use of fast neutrons with their low OER might eliminate this requirement for specially selected fractionation regimens and this would certainly constitute an advantage [9].

An illustration of the fact that in fractionated treatments of tumours the effective gain factor is smaller than the maximum gain factor for the oxygen effect, can be derived from data obtained with the R–1 rhabdomyosarcoma in the rat. For this tumour results are available for impairment of the reproductive integrity of cells irradiated *in vitro* or *in vivo* with a range of doses and dose fractionation regimens [1,10]. It has been shown that cure rates observed after single doses of 6000–7000 rad of X-rays agree well with predictions obtained by extrapolation of the survival curve for clonogenic cells irradiated in the tumour [1]. It was concluded that 90% cures are obtained due to a decrease of the fraction of clonogenic cells to 10^{-9} in tumours containing approximately 10^8 clonogenic cells. From similar extrapolations of data for fractionated treatments, total doses have also been derived, expected to be required for attaining a survival level of 10^{-9} with daily dose fractions of 100, 200, 300, and 400 rad, as well as with other schemes [1]. The results have been used to derive the two "iso-effect" curves shown in Fig. 2(A) as "X-ray dose required" and "neutron dose re-

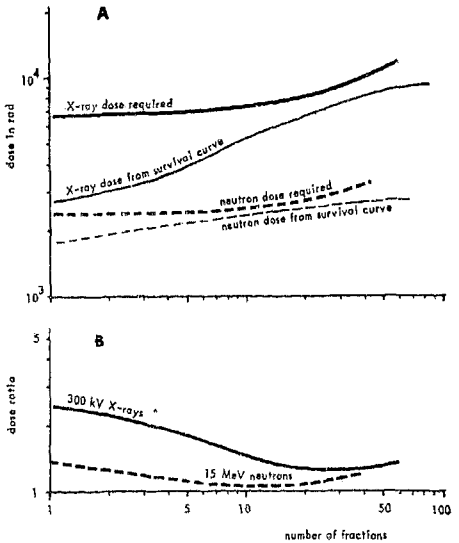


Fig. 2. Relationships between the numbers of daily dose fractions and total doses required to attain a surviving fraction of 10^{-9}*, for R-1 cells irradiated in the tumour and* in vitro*, respectively.* A: *Iso-effect curves for X-rays and* 15 *MeV neutrons derived from irradiation of tumours (thick solid and dotted lines) and calculated from survival curves assuming full repair of sublethal damage between fractions (thin solid and dotted lines).* B: *Ratios of X-ray doses required and calculated and of neutron doses required and calculated.*

quired". These curves differ significantly from the two other curves of Fig. 2(A), which have been calculated on the basis of survival curves for R–1 rhabdomyosarcoma cells irradiated and assayed in culture. For the derivation of these curves it was assumed that no other factors but the intrinsic radiosensitivity and complete repair of sublethal damage in the intervals play a part. Comparison of the two curves for X-rays as well as for neutrons shows that doses required for irradiations of intact tumours are always larger than corresponding doses predicted on the basis of survival curves of cells irradiated *in vitro*.

For single doses and a few large fractions this difference between the two curves for X-rays must be ascribed to the presence of 15% severely hypoxic cells in the tumours [10]. With increasing numbers of fractions, reoxygenation plays an increasingly important role and the difference between the two curves for X-rays becomes smaller. The ratio of the doses of X-rays derived from these curves has been presented in Fig. 2(B) as a function of the dose per fraction, showing a minimum between 20 and 40 fractions. This indicates that with a treatment regimen of 20–40 daily fractions the total dose required for 90% cure is closest to the value derived from simple cell survival curve analysis. The rise of the curves of Fig.2(B) at larger numbers of fractions is due to the increasing influence of proliferation of cells in the intervals [10].

The curves given in Fig. 2(A) for neutrons show similar features as those for X-rays, except that the influence of anoxic cells is smaller due to the lower OER. The curve for 15 MeV neutrons in Fig. 2(B) shows a minimum between 8 and 16 fractions. This implies that for neutrons the most effective treatment is attained for smaller numbers of fractions than for X-rays. Thus with respect to responses of this tumour the optimal treatments to be compared are 20–40 fractions of X-rays and 8–16 fractions of 15 MeV neutrons, respectively. The effective gain factor derived in this way from the minima of the curves of Fig. 2(B) is $1{\cdot}25/1{\cdot}05 = 1{\cdot}2$. This value is considerably smaller than the maximum gain factor for single fractions of 1·7, which is equal to the ratio of the OER values.

Comparison of the effective gain factor of 1·2 with the factor of 1·3 derived earlier from variations of RBE values among tumours due to the differences in intrinsic radiosensitivity, indicates that the presence of anoxic cells and the low OER of neutrons as compared with X-rays, might not be the most important factor determining the possible advantage of fast neutrons in radiotherapy. A more uniform response among various types of tumours due to a smaller dependence on accumulation of sublethal damage might be an at least equally important factor as compared with anoxic cells [9].

DIFFERENCES IN RADIOSENSITIVITY IN THE CELL CYCLE

Variations in radiation responses of mammalian cells depending upon their age in the cycle have been shown to be smaller for high-LET than for low-LET radiations. The OER does not change with cell age, however. If tumour cells could be synchronized by a specific dose-fractionation regimen, and irradiated with each fraction in the most sensitive phase without similar sensitization being induced in cells from normal tissues, then a therapeutic advantage would be largest in the case of X-rays. However, in view of the variations in cell cycle times of cells in tumours and the relatively small dose fractions commonly employed in radiotherapy, a significant advantage

from synchronization for treatments of tumours in patients is unlikely to be obtained in a predictable way. A possible exception might be the accumulation of cells in the sensitive phase before mitosis during low dose rate irradiation as described for the R–1 rat rhabdomyosarcoma [11].

PROLIFERATION OF CELLS IN INTERVALS OF FRACTIONATED TREATMENTS AND THE PRESENCE OF NON-PROLIFERATING CELLS

To a first approximation it can be assumed that the RBE of fast neutrons for effects on tumours will not depend on cell proliferation in intervals between dose fractions or during low dose rate treatments [9–11]. However, if longer overall treatment times would have a favourable influence on the therapeutic margin because of repopulation in normal tissues and if neutrons were most effective with a small number of fractions, then fast neutron treatments might have to be given with intervals in excess of one day, in order to obtain optimal results.

In the case of the experimental R–1 rhabdomyosarcoma it has been shown that with treatments of five fractions of X-rays per week, surviving cells proliferate more rapidly than before treatment, starting after the second week of treatment. Similarly increased cell proliferation rates may occur in normal tissues as well, and much more detailed information is required to evaluate whether a therapeutic advantage can be obtained by selecting specific overall times of treatments for various types of tumours.

In addition to proliferating cells (*P* cells), tumours may contain non-proliferating cells (*Q* cells) which may still be clonogenic and have to be killed if tumour eradication is to be achieved. Little is known of the shapes of survival curves of *Q* cells in tumours and the RBE of neutrons for these cells. For some types of cells in culture the X-ray survival curve of non-proliferating is characterized by a smaller shoulder as compared with proliferating cells [12]. We might then expect relatively low RBE values for fast neutrons for these non-proliferating cells. For other types of cultured cells such a reduction in shoulder width has not been observed, however [12].

An important problem concerning non-proliferating cells is whether in tumours the severely hypoxic cells are *P* cells or *Q* cells. If all severely hypoxic cells were part of the *Q* cell compartment, then after large single doses only *Q* cells would survive, and their radiosensitivity would determine the probability of tumour eradication. This problem is evidently related to the question whether *Q* cells do not proliferate in the tumour because of a lack of sufficient oxygen. Experiments aimed at elucidating this problem have been performed with the R–1 rhabdomyosarcoma. After semi-continuous administration of ^{3}H–TdR to a number of these tumours in order to label all *P* cells, cell suspensions were prepared and assayed for clonogenic capacity *in vitro*. Autoradiographs of these cultures revealed whether the clones were derived from cells that had been labelled in the tumour. *Q* cells in this tumour were shown to be equally capable of proliferation *in vitro* as *P* cells. Preliminary data indicate that among cells surviving after a single dose of 800 rad of X-rays, sufficient to cause a large percentage of the oxygenated cells to be killed, *P* cells and *Q* cells were present in similar proportions as in unirradiated tumours. Thus severely hypoxic cells would be present in the *P* cell compartment as well as in the *Q* cell compartment. In view of these results and the rapid reoxygenation of hypoxic cells in this tumour after irradiation without any shrinkage, the hypothesis might be advanced that hypoxia is not only related to the architecture of the tumour and the blood supply through capillaries, but that the presence of radiobiologically hypoxic cells is related also to an intrinsic property of the tumour cells themselves. The fundamental characteristic of a tumour causing a fraction of the cells to be hypoxic might be related to changes in permeability to oxygen of membranes of subcellular structures, which could lead to temporary lack of oxygen in critical structures during some phase of the cell cycle.

CONCLUDING REMARKS

From the discussion of variations in RBE values of fast neutrons for different types of tumours related to various factors, it can be concluded that considerable differences have been observed. Although human tumours treated by radiotherapists differ from experimental tumours in a number of aspects, e.g. growth rate, growth fraction, homogeneity of the cell population, and in the fact that they have not been selected by transplantation or culture procedures, similarly large differences in RBE values may be expected to be observed in clinical applications. Notwithstanding these uncertainties, the application of fast neutrons

in clinical radiotherapy should proceed because sufficient data on RBE values for experimental systems, especially normal tissues, are available to be used as a first guide. These values will subsequently have to be modified as data from clinical model studies and clinical trials become available. Finally, these investigations must lead to an evaluation of the types of tumour for which high RBE values can result in improvements of cure rates.

REFERENCES

1. G. W. Barendsen, Quantitative biophysical aspects of responses of tumours and normal tissues to ionizing radiations. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard), Vol. 9, p. 101. North-Holland, Amsterdam (1973).
2. G. W. Barendsen, T. L. J. Beusker, A. J. Vergroesen and L. Budke, Effects of different ionizing radiations on human cells in tissue culture. *Radiat. Res.* **13,** 841 (1960).
3. G. W. Barendsen, Dose–survival curves of human cells in tissue culture irradiated with alpha-, beta-, 20-kV X- and 200-kV X-radiation. *Nature, (Lond.)* **193,** 1153 (1962).
4. G. W. Barendsen, Effects of single and repeated low doses of ionizing radiations on the proliferative capacity of human cells in culture. In *Cellular Radiation Biology*, p. 469. Williams & Wilkins, Baltimore (1965).
5. J. F. Fowler, Radiation biology as applied to radiotherapy. In *Current Topics in Radiation Research* (Edited by M. Ebert and A. Howard), Vol. II, p. 303. North-Holland, Amsterdam (1966).
6. 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).
7. 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).
8. R. H. Thomlinson, Reoxygenation as a function of tumor size and histopathological type. In *Proc. Conf. Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*, Carmel, 1969. BNL Report 50203 (C-57), p. 242 (1970).
9. G. W. Barendsen, Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. *Europ. J. Cancer* **7,** 181 (1971).
10. 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).
11. H. B. Kal, G. W. Barendsen and J. J. Broerse, RBE values of 15 MeV neutrons applied at low dose rates. *Europ. J. Cancer* **10,** 293. (1974).
12. G. M. Hahn and J. B. Little, Plateau-phase cultures of mammalian cells. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard), Vol. 8, p. 39. North-Holland, Amsterdam (1972).

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On the Relative Importance of Hypoxic Cells for the Radiotherapy of Human Tumours

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Abstract—*Various aspects are discussed of the problem whether the presence of hypoxic cells in human tumours adversely influences radiotherapeutic cure rates to a clinically significant extent. Subsequently, the relative advantages and disadvantages of various approaches to deal with hypoxic cells in human tumours are analyzed, not only with respect to their usefulness in curing patients but also with respect to possible evidence in support of the importance of hypoxic tumour cells in radiotherapy. Finally, potential advantages of pion beams and heavy ion beams over fast neutron beams and high energy photons are pointed out with respect to their dose distributions in patients.*

DOES THE PRESENCE OF HYPOXIC CELLS IN HUMAN TUMOURS ADVERSELY INFLUENCE RADIOTHERAPEUTIC CURE RATES TO A CLINICALLY SIGNIFICANT EXTENT?

THE SUGGESTION was first put forth by Gray and his associates [1,2] more than a decade ago, that severely hypoxic cells within tumours, because of their reduced radiosensitivity, might survive an otherwise sterilizing dose of radiation and, on being revascularized, recover their clonogenic potential and repopulate the tumour. This thesis was so seductively plausible as to have become almost an article of faith for many investigators. Innumerable laboratory and clinical investigations have been carried out in an effort to confirm the existence of hypoxic cells within tumours and to establish their radiotherapeutic importance. In recent years, however, a number of papers have appeared expressing serious doubts about the clinical relevance of the "oxygen effect" [3–6].

It is conclusively established that the concentration of oxygen dissolved in the tissues strongly influences the sensitivity of all cells to low LET radiations. It is also clear that hypoxic cells do exist in many, if not all, animal tumours [cf. 6, 7]. The presence of foci of necrosis in many human tumours at distances from tumour capillaries which correspond to the expected diffusion distance of oxygen in tissues [8], and polarographic measurements indicating the existence of very low oxygen tensions in some areas within skin and breast tumours [9–11], have also provided strong, albeit indirect, evidence for the existence of hypoxic cells within human neoplasms. However, it has not been established that such hypoxic human tumour cells are clonogenic prior to the start of irradiation, or that they remain both hypoxic and clonogenic to the end of a fractionated course of conventional radiotherapy. Hypoxic cells which have lost their clonogenic potential either prior to or during the course of radiotherapy have lost the capacity to influence radiotherapeutic cure rates.

The sustained presence of hypoxic, clonogenic cells cannot possibly be an important factor in *all* human tumours, or else the doses currently used in radiotherapeutic practice

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should never have succeeded in curing any tumours at all. Yet high primary control rates are consistently achieved for a spectrum of human neoplasms with fractionated total doses on the order of 6000 rad delivered in approximately 30 fractions, corresponding to a nominal single dose, using the Ellis formula [12], of about 1800 ret. It is also known that single doses of 1800 to 2500 rad, used occasionally in the treatment of relatively small skin cancers and at one time in the treatment of oropharyngeal and bladder cancers [cf. 6, 13], have resulted in control rates of 90% or more for the former and substantial control rates for the latter.

Secondly, the available dose–response data for local control of certain early or moderately advanced human tumours yield curves which are much steeper than would be expected if hypoxic cells were a critical factor in their radiotherapeutic response [6]. If significant numbers of hypoxic, clonogenic cells were present throughout the course of radiation, heterogeneity of the radiation response would be expected, with a correspondingly shallower slope for the tumour control probability curve.

Finally, a randomized trial in which patients with osteosarcomas and soft-tissue sarcomas involving the extremities were irradiated under tourniquet hypoxia to doses 2- to 3-fold greater than conventional levels failed to yield the dramatic increase in radiocurability of such lesions that might have been expected had their radioresistance been due to the presence of significant numbers of hypoxic, clonogenic tumour cells [14]. Studies in which hyperbaric oxygen has been used in conjunction with low LET radiotherapy, though still incomplete and controversial, have to date revealed no decisive advantage of this approach. The most optimistic assessment one can make of the situation at this point is that perhaps the circumvention of the hypoxic cell problem may yet contribute to the successful radiotherapy of some human tumours, particularly those which are relatively massive and advanced, but that it is unlikely that the response of most tumours will be significantly altered.

How can we account for the fact that hypoxic, clonogenic cells do not result in radiotherapeutic failure more often than they apparently do? At least four different mechanisms, which are not mutually exclusive, have been proposed:

1. Some of the initially hypoxic cells are recruited into the oxic tumour cell population during the course of fractionated radiotherapy as a consequence of the phenomenon of reoxygenation [7, 15–20];

2. Those cells which remain hypoxic for a sustained interval during fractionated radiotherapy lose their clonogenic potential and thus no longer pose a threat from the radiobiologic standpoint;

3. The decreased capacity for recovery between fractionated doses which is known to occur in severely hypoxic cells [cf. 21] may, in large part, compensate for the relative radioresistance these cells would be expected to have by virtue of their low oxygen concentration. Such cells would have little influence on curability if their recovery between fractions were to partially or completely offset their otherwise greater radioresistance; and

4. Under conditions of conventionally fractionated radiotherapy, small dose increments on the order of 200 rad per day are given. Such doses are close to the shoulder region of the dose–survival curves for cells exposed to X-rays, and thus, as Withers [6] has pointed out, "killing is restricted essentially to the oxic fraction, and dose is not 'wasted' on anoxic cells". During the course of fractionated treatment, the hypoxic, clonogenic cells presumably become reoxygenated, and are then susceptible to killing by the next small fractionated dose.

WHAT ARE THE RELATIVE ADVANTAGES AND DISADVANTAGES OF VARIOUS APPROACHES TO THE PROBLEM OF HYPOXIC CELLS IN HUMAN TUMOURS?

1. *Hyperbaric oxygen*

Because it is time-consuming, cumbersome, somewhat uncomfortable for the patient, and relatively expensive, hyperbaric oxygen has tended to be used in relatively few large-dose fractions, separated by intervals of several days, rather than in conventional daily fractionation schedules [22]. This has made comparison with conventional fractionation difficult, since two variables are involved, oxygen concentration and time–dose fractionation. It has also been reported [23] that hyperbaric oxygen can induce vasoconstriction and impaired tissue circulation; if so, this would prevent the attainment of higher oxygen concentrations in hypoxic zones within tumours and militate against the success of this method.

The only randomized clinical trials in which a significant difference in local tumour control has been observed are those in which relatively few, large dose fractions of radiation were employed. However, these clinical trials were biased by their initial design, since such

sparsely fractionated schedules, though practical for treatments under hyperbaric oxygen, are radiobiologically disadvantageous for radiotherapy during conventional air breathing because fractionation intervals in excess of 24 hr are longer than is required for full reoxygenation of the hypoxic tumour cell population, and the relatively large doses employed with the short fractionation schedules result in much "wasted" irradiation of the hypoxic cell component of the tumour. Conversely, clinical trials in which hyperbaric oxygen and air breathing have been compared under conditions of conventional daily fractionation were probably biased against hyperbaric oxygen. The relevant clinical trial must compare relatively few large-dose fractions delivered under hyperbaric oxygen with air breathing carried out under conditions of conventional fractionation. Such clinical trials are currently under way, but results are not yet available.

2. *Tourniquet hypoxia*

The principal limitation of this approach is that it can be applied only to neoplasms on the extremities and that the degree of hypoxia obtainable may be limited due to the leaching out of oxygen from myoglobin in skeletal muscle. One clinical trial in which osteosarcomas and soft-tissue sarcomas of the extremities were irradiated under tourniquet hypoxia at doses of 12, 14, and 16 krad [14] failed to indicate a higher tumour control rate at the two lower dose levels, in contrast to expectation if hypoxic, clonogenic cells had constituted a significant and persistent component of the sarcoma cell population. The highest dose, 16 krad, did result in a higher tumour control rate, but also produced an excessive incidence of normal tissue injury. The relative rarity of osteogenic sarcomas and soft-tissue sarcomas of the extremities and their proclivity for distant, blood-borne metastasis further diminish the attractiveness of additional clinical trials of tourniquet hypoxia.

3. *Low-dose-rate radiotherapy*

A number of investigators have now shown convincingly that simple reduction of the dose rate of low LET radiations from levels of 10–100 rad or more per min to 10–30 rad per hr results in a decrease in OER roughly comparable to that attainable with neutrons [24, 25]. In practice, such low dose rates would pose serious logistical and staffing problems, since they would greatly reduce treatment capacity per machine, necessitating a considerable and very costly expansion of treatment facilities and of staff. However, to my knowledge, no detailed analyses of the relative cost of this approach vs the provision of comparable capacity for cyclotron neutron beam therapy have been made; it is quite possible that the former would be less expensive than the latter. A more serious objection to the low dose rate approach is that patients with cancer, many of whom are severely debilitated, may not be able to tolerate such very long treatment times.

4. *Oxygen-mimic radiosensitizers*

The chemical nature, mechanisms of action, and potential clinical efficacy of the heterogeneous group of compounds capable of substituting for oxygen have been the subject of extensive study [cf. 26]. The rationale for their use rests on the postulate that, since they are not metabolized like oxygen, they should be capable of diffusing a significantly longer distance from capillaries and could thus reach hypoxic cells within the tumour volume. Until recently, the available agents were either prohibitively toxic or radiobiologically inert *in vivo* or both. However, very encouraging preliminary results have now been obtained with nitrofuran derivatives [27] and especially with Flagyl [cf. 26], a drug already extensively used in man and thus known to have acceptably low toxicity. Adams [26] has made the cogent point that clinical trials of the oxygen mimics would provide the most unambiguous and critical test of the thesis that hypoxic, clonogenic cells play a radiotherapeutically important role in a significant proportion of human neoplasms. However, this will be true only if the waters are not muddied by premature, ill-conceived, and poorly controlled trials of these compounds by radiotherapists in small community hospitals and private offices.

5. *High LET radiations*

Cells irradiated with neutrons, negative pi-mesons, and heavy ions are all known to exhibit a lesser degree of sensitization due to the presence of oxygen, with an OER in the range of 1·5–1·9 as compared with 2·6–3 for low LET radiations such as X-rays [28]. One of the principal difficulties with the use of neutrons has been the unavailability of appropriate radiation sources. Target half-life difficulties and relatively low fluxes have continued to plague the development of D–T neutron generators, and neutron beams produced by large, expensive cyclotrons are currently the only practical source of neutron beams for

clinical radiotherapy. With the small number of such cyclotrons available, it will be many years before a sufficient number of patients can be treated in randomized clinical trials to yield statistically significant comparisons between neutrons and conventional gamma-ray or megavoltage X-ray therapy.

Moreover, as Withers [6] has pointed out, if neutrons did indeed prove superior to X-rays, alternative radiobiological mechanisms for their superiority can be proposed which would leave in doubt the issue of hypoxic, clonogenic cells *per se*. For example, it is known that age-dependent fluctuations in radiosensitivity of cells are of lesser amplitude with high LET radiations than they are with X-rays. Accordingly, if a larger proportion of tumour cells than of normal cells are in radioresistant phases of the division cycle, neutrons would be expected to be relatively more damaging to the tumour than X-rays, and there would be a differentially higher RBE of neutrons, relative to X-rays, for tumour than for normal tissue. There are also data suggesting that the RBE for certain types of tumours, especially those with slow growth rates, are larger than the RBE for normal tissues. Obviously, these possibilities should not negate the attractiveness of clinical trials of neutron-beam radiotherapy. On the contrary, since it may well prove to be true that hypoxic, clonogenic cells have relatively little impact on radiotherapeutic control of tumours, the existence of other possible radiobiologic advantages of neutrons makes such clinical trials somewhat more attractive, even though their ultimate interpretation on radiobiological grounds may be less clear-cut.

The potential advantages of negative pions and heavy ions are not limited to their relatively low OER. These radiations also offer the potential of far superior dose distributions within the volume of the tumour, with a significantly reduced volume of normal tissue in the high-dose region [29,30]. This advantage may well prove to be far more important than the lower OER of the high LET radiations. Indeed, in striking contrast to the failure to date of hyperbaric oxygen or tourniquet hypoxia, the improved dose distribution made possible by the development of megavoltage X- and gamma-ray sources has unquestionably increased the cure rates of a number of clinically important neoplasms, and extended the scope of curative radiotherapy to embrace other types of tumours which were rarely, if ever, cured with kilovoltage X-rays. Thus, since it has already been shown that dose distribution does indeed make a difference, it is not at all unreasonable to anticipate that another "quantum jump" in cure rates may be achieved when the highly sophisticated dose distributions attainable with these particles become available for clinical use.

For many tumours, the dose of megavoltage X- or gamma-rays used in clinical practice is limited by the probability of normal tissue injury to levels well below those which would be optimal for tumour eradication. Since the probability of tissue injury is a composite function of dose, time, and normal tissue volume, a reduction of normal tissue volume encompassed within the high-dose region of pion or heavy ion beams should permit an increment of dose, which, because of the known steepness of the dose–tumour eradication curve, would be expected to yield a significant increase in the probability of tumour eradication [31].

Moreover, as Bond [32] has suggested, the pion dose per fraction could be so chosen that the dose to tumour tissue within the "star" region of the beam would fall on the exponential portion of the high LET dose–survival curve, whereas the dose to normal tissues outside the "star" region would fall in the "shoulder" region of the low LET dose survival curve and thus permit the differential recovery of these normal tissues during the intervals between successive dose fractions. Finally, Kallman [33] has pointed out that the novel multiport design of the Stanford pion channel would confer the unique advantage of differential dose rate. When all 60 channels are utilized, the low LET region along each ray will be irradiated at a dose rate approximately 100-fold lower than that in the high LET region in which all the rays converge. This should further contribute to the sparing of normal tissues outside the high LET region. Thus, the potential of such pion beams rests securely on physical and radiobiologic factors of established clinical importance: dose distribution, differential recovery, and differential dose rate; and the fact that they also offer a relatively low OER, presumably comparable to that of neutrons, is almost an incidental additional advantage.

REFERENCES

1. 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).
2. L. H. Gray, Radiobiologic basis of oxygen as a modifying factor in radiation therapy. *Amer. J. Roentgenol.* **85,** 803 (1961).
3. E. J. Hall, The oxygen effect—pertinent or irrelevant to clinical radiotherapy? *Brit. J. Radiol.* **40,** 874 (1967).
4. H. S. Kaplan, Radiobiology's contribution to radiotherapy: promise or mirage? Failla Memorial Lecture. *Radiat. Res.* **43,** 460 (1970).
5. R. J. M. Fry, E. C. Gregg, R. B. Painter and W. C. Roesch, High-LET radiation in radiotherapy. A report from the Radiation Study Section of the National Institutes of Health. *Radiology* **103,** 215 (1972).
6. H. R. Withers, Biological basis for high-LET radiotherapy. *Radiology* **108,** 131 (1973).
7. R. F. Kallman, The phenomenon of reoxygenation and its implications for fractionated radiotherapy. *Radiology* **105,** 135 (1972).
8. 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).
9. D. B. Cater, A. F. Phillips and I. A. Silver, The measurement of oxidation-reduction potentials, pH, and oxygen tension in tumours. *Proc. Roy. Soc. B* **146,** 382 (1957).
10. D. Jamieson and H. A. S. van den Brenk, Oxygen tension in human malignant disease under hyperbaric conditions. *Brit. J. Cancer* **19,** 139 (1965).
11. H. T. S. Evans and P. F. D. Naylor, The effect of oxygen breathing and radiotherapy upon the tissue oxygen tension of some human tumors. *Brit. J. Radiol.* **36,** 418 (1963).
12. F. Ellis, Relationship of biological effect to dose–time fractionation factors in radiotherapy. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard), Vol. 4, p. 382. North-Holland, Amsterdam (1968).
13. W. Rider, (personal communication).
14. H. D. Suit and R. Lindberg, Radiation therapy administered under conditions of tourniquet-induced local tissue anoxia. *Amer. J. Roentgenol.* **103,** 27 (1968).
15. 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).
16. L. M. van Putten, Tumour reoxygenation during fractionated radiotherapy; studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).
17. R. H. Thomlinson, Changes of oxygenation in tumours in relation to irradiation. *Front. radiat. Ther. Oncol.* **3,** 109 (1968).
18. M. J. Hawkes, R. P. 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).
19. 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).
20. H. D. Suit and J. Schiavone, Effect of a first dose of radiation on the proportion of cells in a mouse mammary carcinoma which are hypoxic. *Radiology* **90,** 325 (1968).
21. T. L. Phillips, Split-dose recovery in anoxic and hypoxic normal and tumor cells. *Radiology* **105,** 127 (1972).
22. I. Churchill-Davidson, C. A. Foster, G. Wiernik, C. D. Collins, N. C. D. Pizey, D. B. L. Skeggs and P. R. Purser, The place of oxygen in radiotherapy. *Brit. J. Radiol.* **39,** 321 (1966).
23. R. J. R. Johnson, A comparison of the effect of hyperbaric oxygen and oxygen plus 5% Co_2 on tissue circulation and oxygenation. *Radiology* **98,** 177 (1971).
24. E. J. Hall, J. S. Bedford and R. Oliver, Extreme hypoxia: its effect on the survival of mammalian cells irradiated at high and low dose-rates. *Brit. J. Radiol.* **39,** 302 (1966).

25. M. Fox and A. H. W. Nias, The influence of recovery from sublethal damage on the response of cells to protracted irradiation at low dose-rate. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard). Vol. 7, p. 71. North-Holland, Amsterdam (1970).
26. G. E. Adams, Electron-affinic sensitizers for hypoxic cells irradiated *in vitro* and *in vivo*: current status. In *Modification of Radiosensitivity in Biological Systems*, International Atomic Energy Agency, Vienna, (In press).
27. J. D. Chapman, A. P. Reuvers, J. Borsa, A. Petkan and D. R. McCalla, Nitrofurans as radiosensitizers of hypoxic mammalian cells. *Cancer Res.* **32,** 2616 (1972).
28. G. W. Barendsen, Responses of cultured cells, tumours and normal tissues to radiations of different linear energy transfer. In *Current Topics in Radiation Research Quarterly* (Edited by M. Ebert and A. Howard, Vol. 4, p. 295, North-Holland, Amsterdam (1968).
29. C. A. Tobias and P. Todd, Heavy charged particles in cancer therapy. *Nat. Cancer Inst. Monogr.* **24,** 1 (1967).
30. M. A. Bagshaw, D. P. Boyd, W. M. Fairbank, H. S. Kaplan, G. C. C. Li, H. A. Schwettman and B. B. Palos, Clinical dosimetry for negative pi mesons. *Radiology* **108,** 197 (1973).
31. H. S. Kaplan, H. A. Schwettman, W. M. Fairbank, D. Boyd and M. A. Bagshaw, A hospital-based superconducting accelerator facility for negative pi meson beam radiotherapy. *Radiology* **108,** 159 (1973).
32. V. P. Bond, Negative pions: their possible use in radiotherapy. *Amer. J. Roentgen.* **111,** 9 (1971).
33. R. F. Kallman, (Personal communication).

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The Effect of Fractionated Doses of Neutrons on C_3H Mouse Mammary Tumours

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Abstract—*The doses required to control 50% of tumours at 150 days after irradiation had previously been determined for three neutron and three X-ray fractionation schedules: single doses, 5 fractions in 9 days (5F/9d) and 9F/18d. The present experiments extend this work to 3F/4d and 15F/18d neutron and X-ray fractionation schedules. The tumour control results were compared with skin reactions caused in the feet of albino mice by the same dose schedules. Little difference in therapeutic effectiveness was found between the neutron and X-ray results, both the neutron and X-ray 3F/4d schedules being good and both 15F/18d schedules being poor.*

INTRODUCTION

THE AIM of our investigation is to relate measurable properties of the C_3H mouse mammary carcinoma, such as its rate of growth, shrinkage and regrowth after irradiation, cell proliferation kinetics, reoxygenation kinetics, and recovery from sublethal injury to the therapeutic effects of fractionated doses of X-rays or fast neutrons. This type of tumour was chosen as a critical test object for neutron therapy because it was known to shrink and reoxygenate rapidly and rather thoroughly after a dose of 1500 rad of X-rays [1], which would of course reduce the advantage of the neutron beam.

In a previous paper [2] the dose required to control 50% of the tumours (the TCD 50) at 150 days after irradiation was determined for six schedules: single doses, five fractions in nine days (5F/9d) and 9F/18d using either cyclotron neutrons or 250 kV X-rays. The six schedules were than compared by determining from experimental dose–response curves the skin reactions on the feet of albino mice for each schedule at the dose corresponding to 50% tumour control.

It was found that several (five) large doses of X-rays given at the optimum interval for reoxygenation in this tumour (2 days; [1]) were as effective as either of the fractionated neutron treatments. Nine smaller fractions of X-rays at the same spacing however (9F/18d) were significantly less effective. There was no such loss of effectiveness in the 9F/18d neutron treatment and it was concluded that although the *best* X-ray and neutron treatments were equally good, the neutron treatments were *less critically dependent upon fractionation* [2]. The "reliability" of neutrons might prove to be a major advantage over X-rays because it is not yet possible to predict optimum X-ray treatments for human tumours.

The present work is an extension of these earlier experiments. The question being asked is: under what conditions might neutron treatments be as good as, better than, or worse than, fractionated X-ray treatments? We need

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to know whether two or three large X-ray fractions at the same optimum spacing are as good as, or better than, the previous best treatment (5F/9d) and whether the same neutron schedules are better or worse than these. We also need to know whether "daily" treatments using more fractions (i.e. 15F/18d) are effective or not. The following schedules were therefore investigated in the present work: single doses, 3F/4d and 15F/18d. Further X-ray schedules were investigated in parallel experiments [3].

MATERIALS AND METHODS

First-generation transplants of spontaneous mammary tumours into C_3H/He mice from the Gray Laboratory inbred colony were used as described in detail elsewhere [2]. Transplants of spontaneous tumours were made weekly into 50–80 mice (one transplanted tumour per mouse); each neutron schedule employed 54 to 111 mice and each X-ray schedule 75–221 mice drawn from up to 20 transplants. The tumours were transplanted subcutaneously onto the chest wall of male or female mice, mainly females.

The irradiation techniques were similar to those previously described [2] except that a 240 kVp Pantak X-ray machine was used with 0·25 mm Cu and 1 mm Al filter giving a HVL of 1·3 mm Cu and a dose rate of 240 rad/minute at the centres of the four tumours which were irradiated simultaneously. The fast neutrons were generated from the MRC cyclotron by allowing the 16 MeV deuteron beam (40–80 μA) to strike a thin beryllium target backed with copper. The neutrons were collimated by steel to provide five beams each 2·5 cm in diameter. Five mice were irradiated simultaneously at a distance of 100 cm where the dose rate was 40–80 rad/min [2]. The tumours hung freely through an oval hole $2 \times 2{\cdot}5$ cm cut from the lead cradle which protected the remainder of the mouse from X-rays. During irradiation all mice were surrounded by oxygen warmed to 25 ± 1°C in order to be comparable with other work [2]. The doses were given "daily" (five times per week excluding weekends) or at "two-day intervals" (Monday, Wednesday and Friday) using equal doses per fraction in each dose group.

Three perpendicular diameters of each tumour were recorded 2–3 times per week until they reached $6{\cdot}5 \pm 1$ mm average diameter at which time they were irradiated. They were measured at approximately weekly intervals thereafter for at least 150 days. If a tumour regrew to larger than 6 mm mean diameter it was scored as a "recurrence"; the mice were usually kept until the tumour reached at least 8–9 mm to be sure the growth was progressive. At 150 days from the mid-time of irradiation tumours under 4 mm in mean diameter were scored as "controlled". Those of 4–6 mm diameter inclusive were scored as ambiguous and deleted from the analysis. They comprised an average of 4·3% (1–10%) of all tumours analysed, with no bias to any one schedule. If a mouse died before 138 days with a "controlled" or "ambiguous" tumour it was deleted from the analysis; some 12% of mice were lost in this way from the fractionated schedules, usually because a spontaneous tumour arose elsewhere in the mouse, occasionally from lung metastases or, rarely except in the single-dose irradiations, from radiation injury to the intestine. However in the single-dose schedules the intestinal deaths amounted to about 17% of all mice irradiated. If a mouse died between 138 and 150 days with a tumour smaller than 2 mm it was accepted as controlled; extrapolation for more than 12 days was not accepted These criteria were somewhat more precise and stringent than those used previously [2] and no problems of interpretation arose.

A significant difference in TCD 50 of about 800 rad (835 ± 158 rad S.E.M.) of X-rays was found between male and female mice in a parallel series of experiments [3], males requiring the higher doses. The neutron experiments employed female mice, except the 15F/18d schedule in which 37/96 mice were male. Although the sex difference in TCD 50 was not significant in the present results, a small correction to the value of TCD 50 was made, on the basis of the simple proportion of male to female mice used, as in the series of X-ray experiments [3]. This TCD 50 was smaller by 5% when corrected to be an equivalent "all-female" value, as done for the present results.

In order to compare the tumour control results with some normal tissue effects, skin reactions caused in the feet of albino mice were used, as in the previous work, except that now WHT/Ht inbred albino mice were used instead of SAS/TO crossbred mice [2]. Skin reactions were recorded up to 40 days after irradiation and the average reactions calculated over the period 8–30 days after single doses or a corresponding period after the fractionated irradiations obtained by matching the leading edges of the curves of skin reaction against time. A comprehensive set of results for all the X-ray schedules used was obtained [4] but it was not

possible to do the same for the neutron schedules. However the corresponding neutron skin reactions could be assessed with consistency from the published curve of RBE versus neutron dose for skin reactions [5] in association with the recently measured X-ray data on WHT/Ht mice [4], using the RBE for each dose per fraction.

RESULTS

The results are shown in Figs. 1 and 2, plotted as tumour control probability (TCP) versus the corresponding average skin reactions caused on the feet of albino mice. The "best" therapeutic schedules are in the upper left part of these graphs, i.e. greatest tumour response for least normal tissue injury. The dotted curves show the previous "best" (5F/9d) and "worst" fractionated X-ray results (9F/18d) from our previous experiments [2]. In that work the two fractionated neutron schedules (5F/9d and 9F/18d) were as good as the best X-ray treatment.

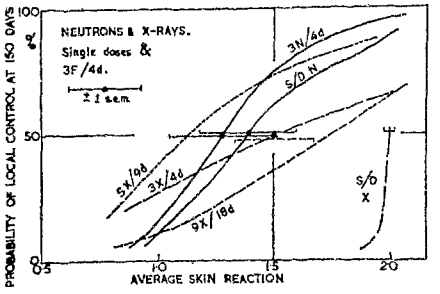


Fig. 1. Probability of local tumour control at 150 days vs. skin reaction. The dotted curves are the "best" and "worst" fractionated schedules from the previous work [2] reassessed according to the present criteria and with respect to WHT mouse skin reactions. 5N/9d and 9N/18d were equally as good as the 5X/9d dotted curve.

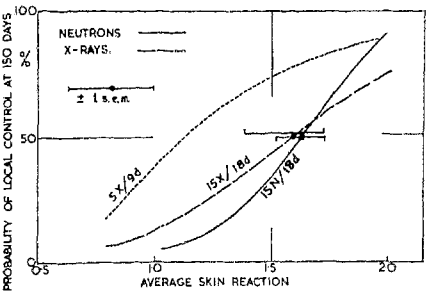


Fig. 2. Probability of local control at 150 days vs skin reactions for both 15F/18d schedules. The dotted line is as in Fig. 1.

Single doses of neutrons in the present work were, as before, much better than single doses of X-rays but not as effective as the best fractionated schedules. The 3F/4d neutron schedule gave better results than the 3F/4d X-ray treatment. The difference is not statistically significant at the TCD 50 level but at higher values of TCP the neutron schedule is significantly better (at TCP of 70% and above, $P \leqslant 0{\cdot}05$). 3F/4d of neutrons at the higher control probabilities is indeed as effective as any other treatment investigated.

With regard to the 15F/18d schedules (Fig. 2) there was no significant difference between the X-ray and neutron results. Both were among the poorest results of any fractionated method tested. Figure 3 summarises these results together with those of other relevant schedules [3]. The "best" treatments are furthest to the left in Fig. 3, i.e. those which caused the lowest average skin reactions at the doses required to control 50% of the tumours.

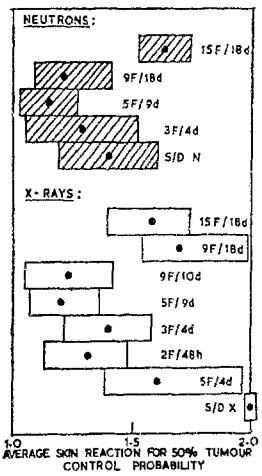


Fig. 3. Mean skin reactions and 70% confidence ranges for the fractionation results reported in this paper, together with previous results [2, 3].

DISCUSSION

Three fractions of neutrons were close to the best results and 3F/4d of X-rays were only slightly worse, not significantly so at the 50% tumour control level. Thus these short fractionation regimes, employing the optimum interval for reoxygenation known from X-ray studies [1] were quite effective, both for X-rays and neutrons. That the 3F/4d neutron results appeared superior, especially at the higher values of TCP, may be due to two factors.

First, the use of large doses at the 2-day optimum interval for reoxygenation interval would enable both the neutron and X-ray 3F/4d treatments to be fairly effective. Second, since reoxygenation is incomplete—although considerable—neutrons can still confer an advantage which is demonstrated more readily when a few large fractions are used than by a larger number of smaller fractions [6].

The poor results with 15F/18d of either neutrons or X-rays suggest that 18 days was too long an overall time for these tumours, whose volume doubling times ranged from 3 to 12 days (average 6 days). The 9F/18d X-ray result was also previously found to be poor. It seems that proliferation can readily overtake cell killing if treatments are prolonged for 18 days, except in the case of the 9F/18 day neutron schedule which was previously found to give good results (Fig. 3). This good result was difficult to explain and a recent reassessment of the previously obtained results, taking the sex of the mice into account, yields a worse value for skin reaction (1·4 average reaction), which lies at the right-hand edge of the shaded rectangle in Fig. 3. A similarity in RBE's for skin reactions and for tumours has also been found for small single doses [8, 9], indicating no therapeutic advantage for neutrons, for this tumour. This similarity is consistent with the reassessed tumour result for 9F/18d just mentioned.

At shorter overall times both the neutron fractionation schedules tested (3F/4d in the present work and 5F/9d previously) appeared therapeutically effective; but the same short X-ray schedules were also effective because the optimum interval for reoxygenation was used. Recent results with other X-ray schedules have confirmed that 5F/9d and 9F/10d were very effective but that 5F/4d of X-rays was poor [3]. The results for 2F/48 hr of X-rays in this tumour were also good, similar to those for a different tumour (RIB5) for two fractions of X-rays or neutrons [10].

It is noteworthy that the neutron result is not worse than the X-ray result for 15F/18d. Indeed, the main impression is one of similarity between the X-ray and neutron results except for poor results with X-rays in the 9F/18d, 5F/4d and single dose schedules. For 3F/4d the neutron results were better than the X-ray results at the higher tumour control probabilities.

CONCLUSIONS

The main advantage of the neutron treatments in this experimental radiotherapy system appears to be not that they give better results than the best of the X-ray treatments, but that they are less likely to give poor results. This tumour system was specially chosen because its ready reoxygenation would be a severe challenge to the relative effectiveness of neutrons. Although X-ray schedules have been found that give as good results as the best neutron treatments (as was predicted from the basic properties of the tumour), it is easier to do badly with X-rays than with neutrons. In experiments with the rat fibrosarcoma RIB_5, which did not reoxygenate so readily, neutrons were considerably more effective than X-rays [10].

It must be emphasised that "daily" fractionation in these tumours is not the same as "daily" fractionation in human tumours with their volume doubling times of weeks or months instead of several days as here. No time-scaling factors can yet be proposed, but the time to achieve optimum reoxygenation and a knowledge of its extent are obvious factors to consider as well as volume doubling time or cell cycle time.

Reoxygenation is expected to be related to the cell loss factor of the tumour, which is in turn expected to be related to the rate of shrinkage after irradiation [11,12]. It may therefore be possible to predict the reoxygenation of human tumours from their gross radiation response and the effectiveness of fast neutrons. Methods of measuring cell loss from tumours using ^{125}IUdR tracer might also be relevant [9].

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REFERENCES

1. A. E. Howes, An estimation of changes in the proportion and absolute numbers of hypoxic cells after irradiation of transplanted C_3H mammary tumours. *Brit. J. Radiol.* **42,** 441 (1969).
2. J. F. Fowler, J. Denekamp, A. L. Page, A. C. Begg, S. B. Field and K. Butler, Fractionation with X-rays and neutrons in mice: response of skin and C_3H mammary tumours. *Brit. J. Radiol.* **45,** 237 (1972).
3. J. F. Fowler, J. Denekamp, P. W. Sheldon, A. M. Smith, A. C. Begg, S. R. Harris and A. L. Page, Optimum fractionation in X-ray treatment of C_3H mouse mammary tumours. *Brit. J. Radiol.* (In press).
4. J. F. Fowler, J. Denekamp, C. Delapeyre, S. R. Harris and P. W. Sheldon Skin reactions in mice after multi-fraction X-irradiation. *Int. J. Radiat. Biol.* **25,** 213 (1974).
5. 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).
6. J. F. Fowler, *Modern Trends in Radiotherapy*—I, (Edited by T. J. Deeley and C. A. P. Wood) Chapt. 8, pp. 145–170, Butterworths, London (1967).
7. A. E. Howes, A. L. Page and J. F. Fowler, The effect of single and fractionated doses of X-rays on the effective proportion of hypoxic cells in C_3H mouse mammary tumours. *Brit. J. Radiol.* **45,** 250 (1972).
8. J. F. Fowler, J. Denekamp and S. B. Field, RBE values for regrowth of C_3H mouse mammary carcinomas after single doses of cyclotron neutrons or X-rays. *Europ. J. Cancer* **9,** 853 (1973).
9. A. C. Begg and J. F. Fowler, A rapid method for the determination of tumour RBE. *Brit. J. Radiol.* **47,** 156 (1974).
10. 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).
11. J. Denekamp, Cell proliferation kinetics in rodent tumours. Ph.D. Thesis, University of London (1968).
12. J. Denekamp, The relationship between the "Cell Loss Factor" and the immediate response to radiation in animal tumours. *Europ. J. Cancer* **8,** 335 (1972).

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Comparison of RBE Values of 15 MeV Neutrons for Damage to an Experimental Tumour and Some Normal Tissues

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Abstract—*A* 15 *MeV deuterium–tritium neutron generator yielding between* 1×10^{12} *and* 3×10^{12} *neutrons per sec was used to measure damage in the EMT6 tumour, bone marrow, oesophagus, and lung of the mouse. RBE and OER values for the tumour are relatively close to those previously reported for the R-1 rhabdomyosarcoma in the rat, the RBE ranging from* 0·3 *at* 50% *survival of hypoxic cells downward as a function of dose and the OER for neutrons being* 1·8. *The RBE's for bone marrow CFU's, oesophageal lethality, and pulmonary lethality were* 1·6, 4, *and* 2·2, *respectively. These results confirm the relative consistency of rodent tumours in response to neutrons and add further evidence of the wide range of RBE's to be expected in normal tissues.*

INTRODUCTION

THE RADIOBIOLOGICAL data that have accrued in the past 10 years on the effects of fast neutrons on tumours and normal tissues suggest that neutrons are probably safe for radiation therapy and that they may have an increased therapeutic ratio (i.e. ratio of tumour damage to normal tissue damage) in certain tumours [1]. It is not yet clear how much the therapeutic ratio might increase with various neutron doses per fraction and for various types of tissues and tumours.

To investigate these questions, we studied a new transplantable rodent tumour that can be assayed by *in vitro* culture [2] and compared it with the bone marrow colony-forming unit (CFU) system, the thoracic irradiation oesophageal LD_{50}, and the thoracic irradiation pulmonary LD_{50} [3].

MATERIALS AND METHODS

The 15 *MeV neutron generator*

The experiments were performed on the Lawrence Livermore Laboratory insulated core transformer deuterium–tritium (D–T) neutron generator. A duoplasmatron ion source produces the deuterons, which are accelerated to an energy of 400 keV and which travel at an angle of 28° to the horizontal towards the target. The target is located in a 40-ft cube area with a low density grid floor in its mid-section under the target to reduce the back-scatter of neutrons. The beam strikes the rotating water-cooled target with a focal spot of less than 1 cm. The maximum beam current on target at the present time is 18 mA. At first a 15-cm dia, rotating, titanium-coated tritium-impregnated target that produced up to 2×10^{12} neutrons/sec was used. This target was replaced in later experiments by a 21-cm dia target of similar construction that produced up to 3×10^{12} neutrons per/sec [4]. The average neutron energy drops from 15·1 MeV at 0° to the deuteron beam to 15·0 MeV at a 30° angle. In the open geometry the gamma dose is negligible.

Irradiation conditions

For the tumour and bone marrow irradiations *in vitro* and *in vivo* an open geometry was used. Plastic thin-walled test tubes containing

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the cell suspension in open or sealed glass ampules were hung from a thin aluminum sheet placed parallel to the direction of the deuteron beam. Hypoxia was created by allowing cellular respiration to remove the oxygen in the sealed ampules. The dose rate ranged from 15 to 125 rad/min.

For the *in vivo* exposures, whole-body irradiation was delivered with the mice placed in perforated plastic tubes 2·5 cm dia and 8 cm in length with a cork in the upper end. These were suspended from another aluminum sheet in the same manner as for the tumour and bone marrow irradiations. In both cases the distance from the source to the samples was between 20 and 63 cm and the dose rates were similar.

For thoracic irradiations with neutrons a shadow-shield collimator was constructed to protect the heads and lower bodies of the mice. The mice were anaesthetized and taped horizontally across the collimator gap so that the entire thorax was exposed and the head and body were shielded.

Neutron dosimetry techniques

In the experiments without collimators, the dose was due exclusively to source neutrons. The primary flux monitor was a proton recoil telescope that served to determine the absolute neutron fluence. In addition, the neutron fluence was measured by using the $^{27}Al(n,\alpha)^{24}Na$ reaction. The two determinations agreed closely. For the conversion of neutron fluence to rad, the value of $6{\cdot}7 \times 10^{-9}$ rad/n/cm^2 was used at 15 MeV [5].

The response to 15 MeV neutrons of a tissue-equivalent chamber filled with air was determined with the recoil telescope. The sensitivity of the tissue-equivalent chamber in rad per nanocoulomb agreed fortuitously to within 1% with the value inferred from the calibration of the instrument using ^{60}Co gamma rays. As a secondary dose monitor, sulphur pellets were activated.

When the collimator was used, additional measurements were performed to determine the changes in neutron spectra, dose, and gamma-ray contribution to the total dose. For this purpose, several different threshold detectors and TLD's were used. Considering both gammas and neutrons, the final shielding factor was 26, based on TLD and activation data, and 21, based on tissue-equivalent ion chambers. The dose rate at the collimator gap in most experiments was 25 rad/min.

Conventional radiation sources

Tumour and bone marrow cells were irradiated with a 2000-Ci self-contained ^{137}Cs irradiator. A rotating turntable was used with cylindrical mouse cages to achieve uniform irradiation. For control thoracic irradiations, the mice were placed in a circle on their backs with $\frac{1}{4}$-in. lead shields over their heads and bodies. They were irradiated with 300 kVp X-rays with a half value layer of 1·6 mm of copper and a TSD of 30 cm. The dose rate in the caesium irradiator was 274 rad/min and in the 300 kVp beam 330 rad/min.

Biological systems

The tumour system used was the EMT6 carcinoma originally developed and described by Rockwell and Kallman [2]. It has been adapted for tissue culture and can be grown as a solid tumour in the animal, grown in culture, cloned from culture, and cloned from preparations of solid tumour in the animal. The tumour is maintained by alternate passages in BALB/c mice and in cell culture. It has been characterized by Rockwell and Kallman [2] as having approximately 30% hypoxic cells and a volume doubling time of about 80 hr at 100 mm^3 decreasing to 5 days at 500 mm^3. After chopping, trypsinization, and suspension it has a plating efficiency of $35 \pm 10\%$ from the solid tumour and of $70 \pm 10\%$ from cultured cells. In these experiments EMT6 carcinomas were irradiated in the flanks of BALB/c mice at a volume of 500–1000 mm^3. Within 2 hr of irradiation the tumours were removed and prepared for culture analysis of surviving fraction.

The bone marrow CFU system used was the one described by McCulloch and Till [6]. In this particular version, the CFU's were irradiated in the donor mouse and transplanted to the recipient mouse within 2 hr after exposure. Surviving fractions were based on the yield of CFU's per 10^5 cells from the femurs of unirradiated animals.

The thoracic irradiason system used has been described elsewhere [3]. After irradiation of a 2·54-cm strip of tissue including the entire thorax, histologic evaluation reveals 2 phases of injury. During the first 28 days severe damage appears in the oesophagus with little or no pulmonary or cardiac change. Between 100 and 160 days severe pulmonary alveolar damage appears with the classic signs of radiation pneumonitis. The patterns of death after irradiation are similar with one wave of death occurring between 8 and 28 days and a second wave between 100 and 160 days. If the mice are made hypoxic by breathing 5·4% oxygen, the early wave of death can be eliminated below 3500 rad but the pulmonary

deaths are unchanged, probably because of exposure of the alveoli directly to the 5·4% oxygen. Animals were observed for deaths within the first 28 days and up to 160 days and the LD_{50} was calculated with a computer program for probit analysis from the percentage of dead mice in each dose group.

RESULTS

The response of EMT6 carcinoma cells *in vitro* is shown in Fig. 1. After neutron irradiation there was no shoulder and the extrapolation number was 1. After caesium irradiation there was an extrapolation number of 7 for both aerated and hypoxic conditions. The RBE in air dropped from 5·3 at a survival of 50% to a minimum of 1·3 based on the D_0 ratios (Table 1). The OER's obtained from these results were constant at any level of survival. The OER based on the D_0 ratio was 1·8 for 15 MeV neutrons and 2·5 for ^{137}Cs.

The response of EMT6 carcinoma cells *in vivo* is shown in Fig. 2. The caesium curve was characterized by an initial shoulder, a sensitive portion with a D_0 of about 160 rad, and a more resistant portion with a D_0 of about 360 rad. This resistant portion has been interpreted as indicating the hypoxic fraction of cells [2]. It could also be explained, at least in part, by a population of cells in a more resistant portion of the cell cycle, although within the limits of the data a slight shoulder and slight resistant portion could be present. From these results the RBE's were 6·3 at 0·5 survival and 2·3 at 0·001 survival. Using the D_0 ratios in the terminal portions of the curves the RBE was 1·2 (Table 2).

For CFU's there was a D_0 of 80 rad with ^{137}Cs irradiation and a D_0 of 50 rad with neutron irradiation. In both cases the extrapolation number was 1. As can be seen from Fig. 3, the neutron results showed considerable scatter.

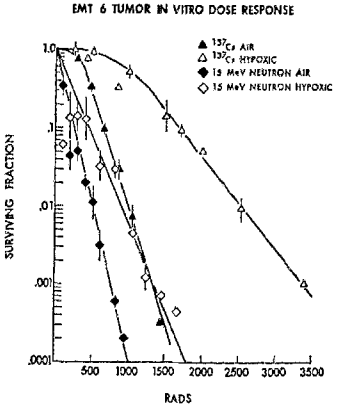


Fig. 1. Dose–response curves for EMT6 tumour cells irradiated in vitro in sealed or open glass ampules with ^{137}Cs gamma rays or 15 MeV neutrons.

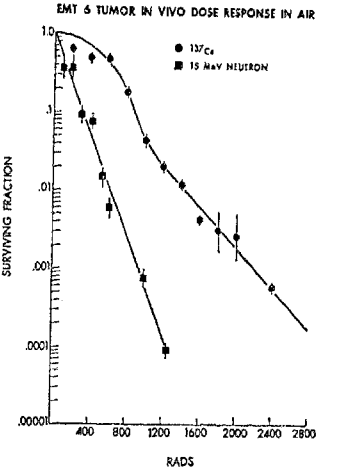


Fig. 2. Response of EMT6 tumour cells irradiated with ^{137}Cs or 15 MeV neutrons in vivo in unanaesthetized BALB/c mice at a tumour volume of 500–1000 mm^3.

Table 1. EMT6 tumour in vitro: RBE values for 15 MeV neutrons vs ^{137}Cs

Aerated*			Hypoxic†		
Survival	Neutron dose (rad)	RBE	Survival	Neutron dose (rad)	RBE
0·5	75	5·3	0·5	150	6·7
0·1	250	2·6	0·1	450	3·7
0·01	500	2·0	0·01	900	2·8
0·001	760	1·8	0·001	1350	2·5

*D_0 ratio: 152/116 = 1·3.
†D_0 ratio: 376/203 = 1·8.

Table 2. *EMT6 tumour* in vivo: *RBE values for* 15 *MeV neutrons vs* ^{137}Cs*

Survial	Neutron dose (rad)	RBE
0·5	90	6·3
0·1	315	2·8
0·01	630	2·3
0·001	940	2·3

*D_0 values: 15 MeV neutrons, 137 rad; ^{137}Cs, 160 rad aerated and 360 rad hypoxic. D_0 ratio (aerated) = 1·2.

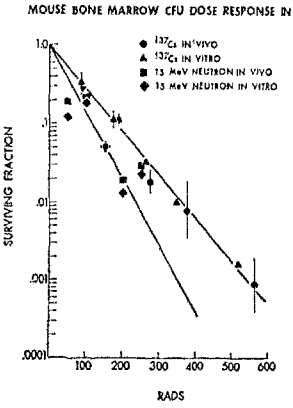


Fig. 3. *Bone marrow CFU response to* ^{137}Cs *or* 15 *MeV neutrons* in vivo *and* in vitro. *Assay carried out in previously irradiated recipients by spleen colony method.*

For the experiments with thoracic irradiation using the 28-day oesophageal endpoint (Table 3) the LD_{50} in air with 300 kVp X-rays was rather high—2682 rad—and there was a very large D_2–D_1. With 15 MeV neutrons the $LD_{50/28}$ was only 658 rad, yielding an RBE slightly above 4. For pulmonary lethality the LD_{50} with mice breathing air was 1339 rad for 300 kVp X-rays and 597 rad for 15 MeV neutrons (Table 4), yielding an RBE of 2·2. It should be pointed out that all but 3 deaths in this group occurred in the first 28 days and were probably due to oesophageal damage. Therefore, the pure pulmonary LD_{50} may be higher for neutrons. In other experiments, hypoxic conditions were used to eliminate the early deaths and in this situation the 300 kVp LD_{50} was 1357 rad. The neutron experiments were done 4 months ago and no deaths were observed during the early period and up to 100 days.

DISCUSSION

When the results of both *in vitro* and *in vivo* irradiation with the EMT6 carcinoma are compared to similar studies done by Barendsen and Broerse with the R–1 rhabdomyosarcoma [7], similar responses are noted. In their *in vitro* studies they found that the OER for 300 kVp X-rays was 2·4 and for 15 MeV neutrons it was 1·4. Both are slightly lower than our values of 2·5 and 1·7, but their method of creating hypoxia was somewhat

Table 3. $LD_{50/28}$ *oesophagus*

Mode	Energy	Number of doses	Dose (95% conf. limits) (rad)	RBE
Air	300 kVp X-ray	1	2682 (2501–2859)	
Air	300 kVp X-ray	2	3527 (3352–3673)*	
Air	15 MeV neutron	1	658 (553– 779)	4
Hypoxia	300 kVp X-ray	1	3908 (3803–3999)	

*$D_2 - D_1$ = 845 rad.

Table 4. $LD_{50/160}$ *lung*

Mode	Energy	Number of doses	Dose (95% conf. limits) (rad)	RBE
Air	300 kVp X-rays	1	1339 (1157–1450)	
Air	15 MeV neutrons	1	597 (486– 702)	2·2
Hypoxia	300 kVp X-rays	1	1357 (1285–1432)	
Hypoxia	300 kVp X-rays	2	1698 (1625–1769)*	

*$D_2 - D_1$ = 341 rad.

different In addition, the D_0 values for both neutrons and X-rays were lower—85 rad vs 116 rad for neutrons and 120 rad vs 152 rad for X-rays and caesium, respectively. The difference between 85 rad and 116 rad for neutrons is too large to be explained on the basis of dosimetry and could be due to differences between the two types of tumour. In the EMT6 tumour the minimum RBE based on D_0 ratios was 1·3. From Barendsen and Broerse's data this value would be 1·4—rather close agreement. As in our results, the RBE is much higher for lower doses and the D_0 change is relatively small. This finding is consistent throughout and indicates that the major difference between neutron and X- or gamma-radiation is the change in the extrapolation number and D_q value. In the case of the EMT6, the extrapolation number changed from 7 to 1, and in the R-1 rhabdomyosarcoma it changed from 10 to 3.

For *in vivo* studies, both Barendsen and Broerse's [7] results and ours show a curve with an apparent resistant fraction for the low LET radiation and a curve without a resistant fraction for the high LET radiation. For the animals breathing air, the D_0 for neutrons was 110 rad for the R-1 sarcoma and 137 for the EMT6 carcinoma. The RBE values for 0·1, 0·01, and 0·001 survival taken from these results are 2·8, 2·3, and 2·3, respectively, in the EMT6 and 2·4, 2·7 and 2·9, respectively, in the R-1. This confirms the elimination of the hypoxic resistant fraction when neutrons are used.

Our results for the bone marrow irradiation suggest that there is little difference between *in vivo* and *in vitro* exposures, so that the presence of bone seems to have little effect on the dose delivered to the cells. This finding and the RBE of 1·6 for neutrons vs gamma rays is close to that reported by Hendry [8].

Our results for thoracic irradiation are puzzling. The RBE of 4 for oesophageal lethality is higher than any previously reported for normal tissues at this neutron dose level, namely 660 rad. It is possible that the relatively high dose for X-ray lethality involves hypoxic cells. Another explanation can be inferred from the data presented by Field and Hornsey [1] for neutron irradiation of the intestine. In their summary a few RBE's between 3 and 4 were shown in the same dose range. Since the oesophagus is part of the gastrointestinal tract, it is possible that this very high RBE is characteristic of that organ. On the other hand, Alper [9] has theorized that the major cause of variation in RBE as a function of neutron dose is the change in the shoulder as expressed by the D_q or the D_2–D_1 value. The very high D_2–D_1 shown for oesophagus, i.e. 845 rad, would be consistent with a very broad shoulder on the dose–response curve and thus a high RBE at moderate neutron doses.

In contrast to the oesophagus, the RBE for lung damage is lower than for most other tissues except bone marrow. However, the results shown here for lung with irradiation in air must be considered tentative since many of the deaths occurred from oesophageal damage. Nevertheless, this represents a maximum value for RBE.

In clinical trials of neutron therapy the data shown here suggest a small advantage for neutrons over gamma rays in tumour killing and also indicate that the oesophagus may be markedly sensitive to neutrons.

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REFERENCES

1. 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).
2. S. Rockwell and R. F. Kallman, Cellular radiosensitivity and tumor radiation response in the EMT6 tumor cell system. *Radiat. Res.* **53,** 281 (1973).
3. T. L. Phillips and L. Margolis, Radiation pathology and clinical response of lung and esophagus. *Front. Rad. Ther. Oncol.* **6,** 254 (1972).
4. R. Booth and H. H. Barschall, Tritium target or intense neutron source. *Nucl. Instrum. Meth.* **99,** 1 (1972).
5. R. L. Bach and R. S. Caswell, Energy transfer to matter by neutrons. *Radiat. Res.* **35,** 1 (1968).
6. 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).

7. 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).
8. J. H. Hendry, The response of haemopoietic colony-forming units and lymphoma cells irradiated in soft tissue (spleen) or a bone cavity (femur) with single doses of X-rays, γ-rays or D–T neutrons. *Brit. J. Radiol.* **45,** 923 (1972).
9. T. Alper, Aspects of neutron therapy based on an analysis of relationships between RBE and dose. *Brit. J. Radiol.* **45,** 39 (1972).

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RBE Values of 15 MeV Neutrons Applied at Low Dose Rates

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Abstract—*Experiments are described in which responses of a solid rhabdomyosarcoma and skin of the rat were compared after irradiation with 15 MeV neutrons and ^{137}Cs gamma rays applied at low dose rates. The RBE of 15 MeV neutrons for damage to the rhabdomyosarcoma was larger by a factor of 1·6 as compared with the RBE for damage to rat skin. This factor is significantly larger than the corresponding values ranging from 1·1 to 1·3 for high dose rates. The implications of these results are discussed with a view of the application of ^{252}Cf neutrons in interstitial and intracavitary radiotherapy.*

INTRODUCTION

THE RELATIVE biological effectiveness (RBE) of fast neutrons for effects on mammalian cells depends in general on the dose rate since the effectiveness of X-rays varies more with dose rate than the effectiveness of fast neutrons. This variation of RBE with dose rate is of interest for fundamental as well as for practical reasons, because differences might be revealed among tumours or normal tissues which may influence therapeutic margins. In addition to neutrons from accelerators used for external irradiation, ^{252}Cf emitting neutrons at low intensities, is being employed for interstitial and intracavitary radiotherapy and experimental data on effects in tumours and normal tissues are required to guide clinical practice.

In the present paper experiments are described in which a solid rhabdomyosarcoma and skin of rats were irradiated with 15 MeV neutrons applied at low dose rates.

MATERIALS AND METHODS

Rat rhabdomyosarcoma

The origin and growth characteristics of the transplantable rhabdomyosarcoma have extensively been described [1, 2]. This tumour consists of cells which after excision of the tumour and application of a cell dispersion technique [3] can be cultured directly *in vitro*. This technique provides the possibility of assaying changes in the fraction of clonogenic cells in tumours after various types of treatment [1].

Rat skin

A skin transplantation method was developed [4] for the assessment of radiation-induced damage to the skin. Following total-body irradiation of WAG/Rij rats two circular white pieces of dorsal skin from each animal were transplanted onto the backs of unirradiated brown (WAG/Rij × BN/Bi)F_1 hybrid rats, in which graft beds had been prepared by excision of similar pieces of skin. After about two weeks the irradiated skin grafts start to decrease in size and, 3–5 months after the irradiation, the ratios of remaining areas of grafted skin relative to controls approach constant values which depend on the dose. The ratio of the treated and untreated areas measured 5 months after transplantation has been used as a parameter for skin damage.

Irradiation techniques

Acute irradiations of tumours with X-rays were carried out with a Philips–Müller X-ray

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generator (300 kV, 10 mA, 340 rad/min, HVL 2 mm Cu, FSD 20 cm). For acute irradiation of the skin the animals received a total-body exposure at a dose rate of 40 rad/min (FSD 60 cm).

Continuous gamma irradiations of tumours (dose rates 75, 110 and 150 rad/hr) and of rat skin (dose rate 85 rad/hr) were carried out with a ^{137}Cs gamma source of 1200 Ci. The animals were given a total-body irradiation. Acute and protracted neutron irradiations were carried out with a Van de Graaff accelerator which accelerates deuterons to produce monoenergetic neutrons of 15 MeV energy through the ^{3}H(d,n)^{4}He reaction.

RESULTS

Survival of rhabdomyosarcoma cells after acute X-ray and 15 MeV neutron irradiation

Results of determinations of fractions of surviving rhabdomyosarcoma cells in tumours treated with 15 MeV neutrons at a dose rate of 20 rad/min and 300 kV X-rays at 340 rad/min are shown in Fig. 1(A), curves 1 and 2, respectively. The points represent mean values of at least three tumours assayed separately [1]. Curve 2 is characteristic for a population of cells consisting of a large fraction of oxygenated cells (85%) and a small fraction of severely hypoxic cells (15%). At doses in excess of 1500 rad the curve can be represented by a D_0 of 305 rad and an extrapolation number (N) equal to 1·5. Curve 1 can be described by a D_0 value of 110 rad and an N value of 1·5. The RBE of 15 MeV neutrons relative to X-rays depends on the dose given; at a survival level of 10 and 0·1%, the RBE values are 2·55 and 2·90, respectively.

Survival of rhabdomyosarcoma cells after protracted ^{137}Cs gamma ray and 15 MeV neutron exposures

Figure 1(B) shows results of determinations of fractions of surviving tumour cells after continuous irradiation with 15 MeV neutrons at a dose rate of 48 rad/hr (curve 1) or with ^{137}Cs gamma rays at dose rates of 75, 110, and 150 rad/hr (curve 2). The points of the curves represent results from assays of individual tumours. A cell yield factor, i.e. the number of cells per gram tumour at the end of the protracted exposure relative to the number of cells per gram of an untreated tumour, has been used to arrive at a correction factor to eliminate influences of cell proliferation and cell loss during protracted exposure [5]. The survival curves which were obtained with the continuous gamma exposure at 75, 110, and 150 rad/hr, respectively, are not significantly different. For the combined data a survival curve has been calculated with the least squares method and the number of clones as a weighting factor. Curve 2 can be described by a D_0 value of 583 (550–615) rad and an N value of 0·94 (0·5–1·7). Curve 1 can be described by a D_0 value of 122 (114–130) rad and an N value of 1·5 (0·7–3·0). The RBE of 15 MeV neutrons at low dose rate relative to X-rays is 3·65 ± 0·1 (assuming an RBE of 0·85 for ^{137}Cs gamma rays). This value is almost independent of the survival level, since both low dose rate survival curves have extrapolation numbers close to I. The RBE of 15 MeV neutrons at low dose rate for the rhabdomyosarcoma is larger by a factor

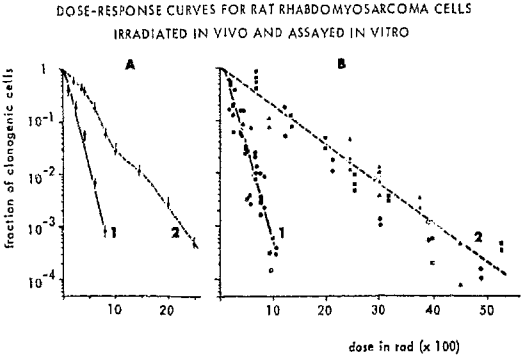


Fig. 1. Survival curves obtained by irradiation of rat rhabdomyosarcomas. A, curve 1: tumours irradiated with 15 MeV neutrons at a dose rate of 20 rad/min; curve 2: tumours irradiated with 300 kV X-rays at a dose rate of 340 rad/min. B, curve 1: tumours irradiated with 15 MeV neutrons at a dose rate of 48 rad/hr; curve 2: tumours irradiated with ^{137}Cs gamma rays at a dose rate of 75◆, 110■, and 150▲ rad/hr.

of 1·3 as compared with the RBE at high dose rate. The efficiency of the protracted exposures is less as compared with acute exposure due to repair of sublethal damage but this effect is partly balanced by a reoxygenation and a synchronization process [5]. These factors influence the response to X-rays stronger than those to fast neutrons. The dose rate effectiveness ratio of the gamma exposure is 1·45 (in which an RBE of 0·85 of gamma rays relative to X-rays is assumed). For 15 MeV neutrons this dose rate effectiveness ratio is about 1·1.

Shrinkage of transplanted skin area after acute X-ray and 15 MeV neutron exposures

Figure 2(A) shows relationships between the area of remaining skin relative to controls, and the dose for acute 15 MeV neutron and 300 kV X-ray exposures applied at dose rates of 20 rad/min and 40 rad/min, respectively. The RBE of 15 MeV neutrons for shrinkage of rat skin area relative to X-rays at the 10% level is 1·65. This level of 10% is obtained at a single dose of about 1760 rad of X-rays, approximately equal to the Ellis' nominal single dose for normal human tissue tolerance.

Shrinkage of transplanted skin area after protracted ^{137}Cs gamma and 15 MeV neutron exposures

Results of determinations of the mean fractional area of skin relative to controls as a function of dose for 15 MeV neutrons administered at 48 rad/hr and ^{137}Cs gamma rays at 85 rad/hr are shown in Fig. 2(B), curves 1 and 2, respectively. Mainly due to repair of sublethal damage, the efficiency of the protracted exposures is less as compared with acute exposure. The sparing effect is larger for gamma rays at low dose rate relative to acute X-ray exposure as compared with 15 MeV neutron exposures at low and high dose rates, respectively. The dose rate effectiveness ratios are 1·75 (in which an RBE of 0·85 for gamma rays relative to X-rays is assumed) and 1·25, respectively. The RBE of 15 MeV neutrons relative to gamma rays at low dose rate is 2·65. With the RBE of 0·85 for gamma rays relative to X-rays the RBE is calculated at 2·25. This RBE value at low dose rate for rat skin is larger by a factor of 1·35 as compared with the RBE value for acute fast neutron irradiation.

Therapeutic gain factor

For fast neutron irradiations at high dose rates the therapeutic gain factor (TGF), i.e. the ratio of the RBE for damage to tumours and the RBE for damage to normal tissues, in the dose per fraction region of clinical interest, is in the range of 1·1 - 1·3 [6]. The TGF for protracted fast neutron irradiation as calculated from Fig. 1 and 2 for the rhabdomyosarcoma and skin, is about 1·6. This value is significantly higher than

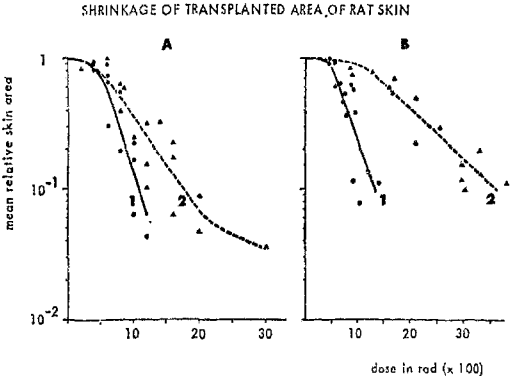


Fig. 2. Dose–effect relationships for reduction of the area of skin grafts. A, curves 1 and 2: dose–effect relationships for 15 MeV neutrons at a dose rate of 20 rad/min and 300 kV X-rays at a dose rate of 40 rad/min, respectively. B, curves 1 and 2: dose–effect relationships for 15 MeV neutrons applied at a dose rate of 48 rad/hr and ^{137}Cs gamma rays administered at a dose rate of 85 rad/hr, respectively.

the published TGF values derived for acute fast neutron irradiation.

DISCUSSION

The basic requirement for fast neutrons to provide an advantage in clinical applications is that the RBE for damage to the tumour is larger than for normal tissue tolerance. Com-

parison of RBE values for the R-1 rhabdomyosarcoma and for normal tissues reported earlier [6] shows that the TGF of about 1·2 for high dose rate irradiations is augmented by about 30% to 1·6 if 15 MeV neutrons are applied at low dose rates. Although the treatment of patients by irradiation at low dose rates poses significant problems, the increase in cure rate from such treatments without increase in normal tissue response might be quite significant. For low-LET radiation an increase in effective dose of 10% caused the cure rate to rise from 20% to 50% for mammary carcinoma in C3H mouse, and an increase in effective dose of 20% caused local control of supraglottic squamous carcinoma to increase from 20% to 80%. For stages 3b and 4 of the cervix carcinoma such a steep rise was not shown, however [7].

Evaluation of radiation effects of ^{252}Cf neutrons on normal tissues have in general resulted in RBE values between 4·1 and 6·6 depending on the end-point and the dose rate. Dean *et al.* [8] found an RBE of 4·1 for $LD_{50/30d}$ in mice at a ^{252}Cf neutron dose rate of 64 rad/hr $(n+\gamma)$ and Atkins *et al.* [9] an RBE of 6·6 for damage to pig skin at a ^{252}Cf neutron dose rate of 12 rad/hr $(n+\gamma)$. RBE values of ^{252}Cf neutrons derived for solid tumours are not yet known. The RBE values derived from the experiments described here can be extrapolated to RBE values of ^{252}Cf if the differences in neutron energy and dose rates are taken into account. For T-1g cells Broerse *et al.* [10] have shown that at low doses the RBE of fast neutrons as a function of mean neutron energy varies from 2·9 for 15 MeV neutrons to 6·1 for 1 MeV fission neutrons. The mean neutron energy of ^{252}Cf is about 2·3 MeV. From an analysis of these data a factor of approximately 1·85 can be derived when a comparison between RBE values of 15 MeV neutrons and ^{252}Cf neutrons will be made.

The RBE of 2·65 relative to gamma rays for shrinkage of skin area can then be extrapolated to an RBE value of 4·9 at a neutron dose rate of 48 rad/hr. This value is similar to the reported RBE values for normal tissues. If the extrapolation is carried out for the rhabdomyosarcoma, an RBE value of about 8 is obtained. It will be clear, however, that this extrapolation depends on various assumptions and more data have to be obtained for the responses of normal tissues and other experimental tumours to protracted neutron irradiation before definite conclusions can be drawn about a possible therapeutic advantage of continuous neutron irradiation at low dose rates.

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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. A. F. Hermens and G. W. Barendsen, Cellular proliferation patterns in an experimental rhabdomyosarcoma in the rat. *Europ. J. Cancer* **3,** 361 (1967).
3. H. S. Reinhold, A cell dispersion technique for use in quantitative transplantation studies with solid tumours. *Europ. J. Cancer* **1,** 67 (1965).
4. 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. 293. North-Holland, Amsterdam (1968).
5. H. B. Kal and G. W. Barendsen, Effects of continuous irradiation at low dose-rates on a rat rhabdomyosarcoma. *Brit. J. Radiol.* **34,** 252 (1972).
6. G. W. Barendsen, Cellular responses determining the effectiveness of fast neutrons relative to X-rays for effects on experimental tumours. *Europ. J. Cancer* **7,** 181 (1971).
7. J. F. Fowler, Summary of general discussion on radiotherapeutic aspects of the clinical application of fast neutrons. *Europ. J. Cancer* **7,** 259 (1971).
8. F. K. Dean, S. C. Bushong, N. Prasad and S. Briney, The relative biological effectiveness of californium-252 on acute mammalian lethality. *Radiology* **104,** 699 (1972).
9. H. L. Atkins, R. G. Fairchild and J. S. Robertson, Comparison of irradiation by californium-252 and radium on the skin of swine. *Radiology* **96,** 161 (1970).
10. 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.* **6,** 559 (1968).

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RBE and OER Values as a Function of Neutron Energy*

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Abstract—*A new set of RBE and OER measurements are reported for ten neutron energies between 60 keV and 15 MeV obtained at the Radiological Research Accelerator Facility at Brookhaven National Laboratory. In addition two higher energy neutron beams were studied at the Naval Research Laboratory in Washington and at the Texas A&M Cyclotron. Two biological test systems were used, namely growth inhibition of* Vicia *seedlings and hamster cells cultured* in vitro. *In the case of the OER, a minimum value of 1·3 was observed for neutron energies of 220–440 keV. OER increased with neutron energy to a maximum of 1·8 for 15 MeV neutrons. A slightly lower value of 1·6 was found to apply to both of the high energy cyclotron-produced neutron beams currently being introduced for radiotherapy. The plot of RBE as a function of neutron energy has a similar shape for both biological test systems employed; the maximum RBE occurs at a neutron energy of about 350 keV. The experimental data are consistent with the theoretical predictions made on the basis of microdosimetric measurements.*

INTRODUCTION

THE RADIOBIOLOGICAL properties of neutrons are of interest from two points of view. First, such data contribute to our basic knowledge of the mechanism of radiation effects. Second, there is the more pragmatic reason that neutrons are being used increasingly in clinical radiotherapy as a result of the encouraging experiences reported from the Hammersmith Hospital.

Two extensive series of experiments have previously been reported in which RBE and OER were studied as a function of neutron energy [1–3].

The present paper describes a new series of measurements that cover a much wider range of energies than has previously been possible. Using the Radiological Research Accelerator Facility (RARAF) at Brookhaven National Laboratory, ten neutron energies have been studied ranging from 60 keV to 15 MeV. The characteristics of the beams used are summarized in Table 1. By using suitable targets essentially monoenergetic neutron beams were generated ranging from 15 MeV down to 220 keV; in addition two lower energy beams were used which consisted of a wide spectrum of energies and are described as the 110 and 60 keV spectra, which refers to their maximum values. High energy neutron beams were studied at the Texas A&M Variable Energy Cyclotron (TAMVEC) and the Naval Research Laboratory (NRL) Cyclotron situated in Washington, D.C. Both of these machines use the *d*-Be process and generate beams containing a wide spectrum of neutron energies with maxima of 50 and 35 MeV respectively.

METHODS

Two radiobiological test systems were utilized. Seedlings of *Vicia faba* were used to determine values for the OER and also to measure the RBE over the low dose range.

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Table 1. *Characteristics of neutron beams studied*

Source	Energy	Max. energy spread	Production process	Average dose/rate For *Vicia*	Average dose/rate For cells
TAMVEC	50 MeV max.	50–zero	d→Be	70 rad/min	70 rad/min
NRL	35 MeV max.	35–zero	d→Be	50 rad/min	50 rad/min
RARAF	15 MeV	±4%	d→T	280 rad/hr	1000 rad/hr
	6 MeV	±7%	d→D	450 rad/hr	475 rad/hr
	2 MeV	±5%	p→T	140 rad/hr	450 rad/hr
	1 MeV	±11%	p→T	70 rad/hr	625 rad/hr
	660 keV	±13	p→T	—	500 rad/hr
	440 keV	+22% −18%	p→T	40 rad/hr	300 rad/hr
	350 keV	+28% −23%	p→T	30 rad/hr	250 rad/hr
	220 keV	±30%	p→T	16 rad/hr	130 rad/hr
	110 keV spectrum	110–zero	p→T	35 rad/hr	85 rad/hr
	60 keV spectrum	60–zero	p→T	15 rad/hr	

The endpoint scored was growth inhibition of the primary root [4, 5]. V79 Chinese hamster cells cultured *in vitro* was the test system of choice to determine RBE's over the higher dose range, and also to obtain information concerning the shape of the survival curves for different neutron energies. Methods of culture have previously been reported in detail [6].

Absolute dosimetry was performed using a 6 mm diameter spherical multiplication ionization chamber constructed of tissue-equivalent plastic. The gamma ray contamination in the beam was measured with a calibrated Geiger–Müller dosimeter. A member of the RARAF staff performed all dosimetry measurements at both outside facilities, as well as at RARAF, using the same equipment.

RESULTS AND DISCUSSION

Figure 1 is a plot of the oxygen enhancement ratio as a function of neutron energy. There is a complex relationship between these quantities.

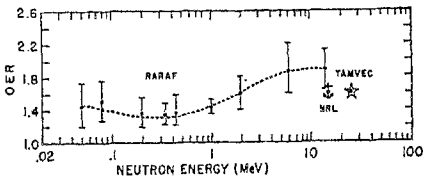


Fig. 1. *OER as a function of neutron energy. The test system used was growth inhibition of* Vicia *seedlings. The closed circles represent data obtained with essentially monoenergetic neutron beams at the Radiological Research Accelerator Facility and has previously been published* [8]. *The vertical bars represent* 95%*-confidence intervals. The anchor and star represent data obtained at NRL and TAMVEC with high energy cyclotron produced neutrons of* 35 *and* 50 *MeV respectively.*

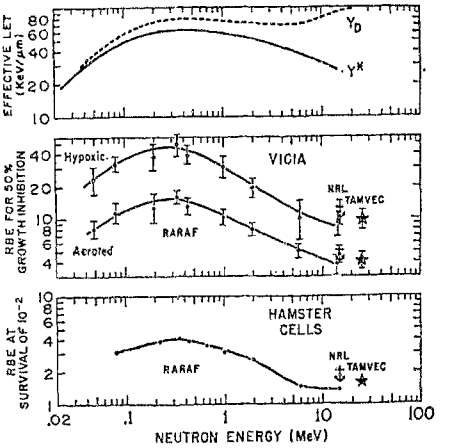


Fig. 2. *Upper panel: The effective LET as a function of neutron energy calculated from microdosimetric data* [7].

Middle panel: RBE as a function of neutron energy measured with Vicia *seedlings under aerated and hypoxia conditions* [8].

Bottom panel: Corresponding data for mammalian cells in culture.

A low OER of about 1·3 is evident for neutron energies of 220–440 keV. As the neutron energy is increased the OER rises to a maximum of 1·8 for 15 MeV neutrons. It has a somewhat lower value for the high energy cyclotron produced neutrons at TAMVEC and NRL, which is of considerable interest for radiotherapy, since such beams already have the attractive properties of a high output and excellent depth dose characteristics. It must be emphasized that in the case of the high energy beams, a wide spectrum of neutron energies is involved and in Fig. 1 the OER is plotted

against the *modal* energy. By contrast all but the two lowest energies at RARAF are monoenergetic. There is at least a suggestion that the OER rises again for the two lowest energy neutron beams utilized. The accuracy of the data is not sufficient for this to be argued with statistical validity, but the trend would be expected from a consideration of the spectra of recoil protons produced.

Figure 2 is a plot of the RBE as a function of neutron energy. Included in this plot are the data for *Vicia* seedlings under both aerated and hypoxic conditions, as well as the corresponding data for Chinese hamster cells irradiated *in vitro*. Also shown are the theoretical predictions of effective LET made by Kellerer and Rossi and based upon microdosimetric data [7]. The general shape of all these curves is similar, corresponding to a maximum RBE at a neutron energy of about 350 keV. The RBE's of the TAMVEC and NRL beams are higher than for 15 MeV monoenergetic neutrons. In both cases the beams include a wide range of neutron energies, and it is an arbitrary decision to plot the RBE values against the *modal* energy.

Acknowledgements—The data summarized in this paper represent the team efforts of many individuals in the Radiological Research Laboratory at Columbia University, and would not have been possible without the help and collaboration of the staffs of TAMVEC and the NRL cyclotrons.

REFERENCES

1. R. J. Berry, Hypoxic protection against fast neutrons of different energies—A review. *Europ. J. Cancer* **7,** 145 (1971).
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, p. 293. North-Holland, Amsterdam (1968).
3. G. W. Barendsen and J. J. Broerse, Dependence of the oxygen effect on the energy of fast neutrons. *Nature, (Lond.)* **212,** 722 (1966).
4. E. J. Hall, The relative biological effectiveness of X-rays generated at 220 KVp and gamma rays from a cobalt-60 teletherapy unit. *Brit. J. Radiol.* **34,** 313 (1961).
5. E. J. Hall and J. Cavanagh, The oxygen effect for acute and protracted radiation exposures measured with seedlings of *Vicia faba. Brit. J. Radiol.* **40,** 128 (1967).
6. E. J. Hall and S. Lehnert, The biophysical properties of 3·9 GeV nitrogen ions. IV. OER and RBE determinations using cultured mammalian cells. *Radiat. Res.* (In press).
7. A. M. Kellerer and H. H. Rossi, The theory of dual radiation action. In *Current Topics in Radiation Research,* (Edited by M. Ebert and A. Howard), Vol. 8, p. 85. North-Holland, Amsterdam, (1972).
8. E. J. Hall, H. H. Rossi, A. M. Kellerer, L. Goodman and S. Marino, Radiobiological studies with monoenergetic neutrons. *Radiat. Res.* **54,** 431 (1973).

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The Response of Chinese Hamster Cells to ^{252}Californium Neutrons and 14 MeV Neutrons

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Abstract—*Chinese hamster (ovary) cells have been exposed to protracted neutron radiation from ^{252}californium at 15 rad/hr and ^{60}cobalt gamma rays at 100 rad/hr under aerated and hypoxic conditions. The response has also been measured to 14 MeV neutrons at 30 rad/min and to 300 kVp X-rays at 100 rad/min. Dose–response data have been fitted to survival curves and values for oxygen enhancement ratio (OER) were derived by comparison of the exponential slopes of the respective curves. The OER for X-rays was 3·2; for 14 MeV neutrons 1·6; for ^{252}californium 1·9, and for ^{60}cobalt 1·4. From these values with these mammalian cells it can be concluded that while a change from acute X-irradiation to acute neutron irradiation would reduce the OER by a factor of 2, no advantage due to a reduced OER would be obtained by changing from protracted gamma irradiation to protracted ^{252}californium irradiation.*

INTRODUCTION

ALTHOUGH Sir James Chadwick received his Nobel prize in 1935 for the discovery of the neutron, this uncharged particle is still not being used routinely in radiotherapy. The main biological advantage of neutron therapy [1] lies in a reduction in the oxygen enhancement ratio (OER) of neutrons for damage in irradiated tumour tissues which are presumed to include a significant proportion of hypoxic cells. The OER is reduced as the ionisation density is increased so that while a useful reduction has been found with fast neutrons with an energy range around 14 MeV, an even greater reduction might be expected with lower energy neutrons [2] and this should apply to those emitted by ^{252}californium.

This hypothesis has been tested using Chinese hamster cells in monolayer culture. Dose-response curves have been obtained for cells irradiated under aerated and hypoxic conditions using protracted ^{60}cobalt irradiation in comparison with that from ^{252}californium neutrons, and acute 300 kVp X-irradiation in comparison with that from 14 MeV D–T neutrons.

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METHODS

Chinese hamster, ovary (CHO) cells were obtained from logarithmic phase monolayer cultures grown in medium F12 supplemented with 10% calf serum. Cells were irradiated at 20°C in suspensions which were dilute for the aerated condition and concentrated for the hypoxic condition. The cell suspensions were then suitably diluted and assayed for survival of colony forming ability (see [3] for details).

Cell suspensions were exposed to protracted irradiation either in the centre of a circular array of ^{252}californium sources where the dose rate was 15 rad/hr, or at the distance from a ^{60}cobalt source where the dose rate was 100 rad/hr. Cells received acute irradiation in the beam of a 14 MeV D–T neutron generator where the dose rate was 30 rad/min, or in the beam of a 300 kV X-ray machine (HVL 2 mm

Cu) where the dose rate was 100 rad/min. The methods of dosimetry and other details have been described previously [3,4]. All statements of neutron dose refer to the total (neutron + gamma) dose.

RESULTS

The survival data from repeated experiments were pooled and the mean values (with their standard errors) are shown in Figs. 1 and 2. Dose–response curves were fitted to these data using the computer programme of Gilbert [5] to obtain the best fit and these curves are shown plotted in Figs. 1 and 2. The parameters of these dose–response curves are presented in Table 1 together with those from X-irradiation data [6] which provide reference values for comparison.

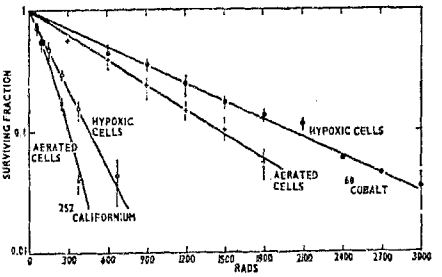


Fig. 1. Cell survival curves for CHO cells irradiated with ^{252}californium or ^{60}cobalt, in air or under hypoxic conditions.

Of the four curves in Fig. 1, three have been fitted to a pure exponential but the ^{252}californium curve for aerated cells has a shoulder before becoming exponential. This means that oxygen is not purely dose-modifying in that comparison (i.e. californium air/hypoxia) and an arbitrary value for OER has been derived from the ratio of the final exponential slopes of the two survival curves. Since both the ^{60}cobalt curves fit pure exponentials the OER is constant at all dose-levels. With the fast neutron curves in Fig. 2 computation indicated no significant difference between their extrapolation numbers and curves were therefore fitted to a common value; the OER is then constant at all dose levels. The X-ray curves [6] were different in shape because there was a significant difference between the values of the extrapolation numbers so that, as with the californium data, a value for OER has been derived from the ratio of the final exponential slopes of the two survival curves. All these numerical data are summarised in Table 1 where protracted irradiation from ^{60}cobalt and ^{252}californium sources is described as low dose rate as distinct to the relatively high dose rate of the X-ray and fast neutron beams.

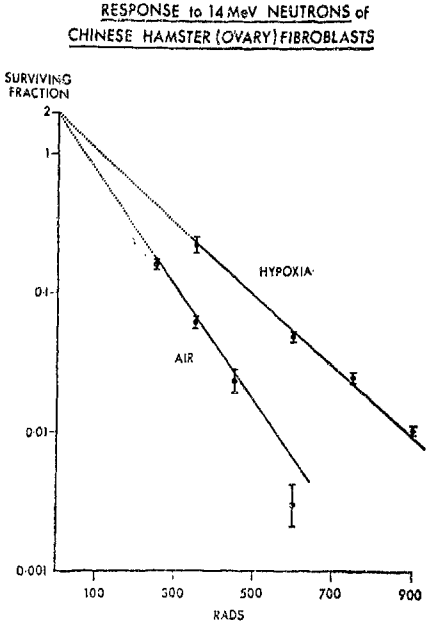


Fig. 2. Cell survival curves for CHO cells irradiated with 14 MeV monoenergetic neutrons in air or under hypoxic conditions (unpublished data of Nias, Greene and Major).

DISCUSSION

The values for OER (i.e. the D_0 ratios) in Table 1 lead to the simple conclusion that, with reference to the value of 3·2 for X-rays at high dose rate, a therapeutic gain would be obtainable if any of the other three irradiation conditions were to be employed instead. As far as the change to fast neutrons at high dose rate is concerned, these data only confirm the general trend reviewed by Duncan [1] that values for OER fall from 2·5 or above with X-irradiation to around 1·5 with fast neutrons. In an earlier comparison of the responses to medium voltage X-rays and 14 MeV neutrons of Chinese hamster cells, Hall [7] reported a fall in OER from 2·67 to 1·5, although this was a different cell line (Chinese hamster, lung). Hall *et al.* [8] have since compared the response of such cells to radium and ^{252}californium at total dose rates of 50 and 18 rad/hr respectively. Their OER values differ from those reported here, being 2·0 for radium and 1·42 for ^{252}californium.

The discrepancy between the data may simply be due to a difference between the lines of Chinese hamster cells, but other reasons may

Table 1. Parameters of CHO cell survival curves

Irradiation conditions	Air D_0	Air N	Hypoxia D_0	Hypoxia N	D_0 ratios
High D/R X-ray	200	3·00	640	1·67	3·2
Low D/R ^{60}cobalt	635	1·00	870	1·00	1·4
Low D/R ^{252}californium	105	1·76	200	1·00	1·9
High D/R neutron	105	1·87	170	1·87	1·6

also be adduced. One of these concerns radiobiological phenomena involved in the change from high to low dose rate [9]. Bedford and Hall [10] had previously observed a significant fall in OER from 2·4 to 1·6 with HeLa cells when the X-ray dose rate was reduced from 103 rad/min to 30 rad/hr. In Table 1 the OER for low dose rate ^{60}cobalt irradiation is 1·4, which is slightly lower than Bedford and Hall [10] value for HeLa cells. But that, in turn, is considerably lower than their present value of 2·0 for Chinese hamster cells [8]. This is now the lowest OER for protracted gamma irradiation in their review of the literature where the data of Bedford and Hall [10] are not immediately relevant. The question remains whether a fall in the dose rate of X- or gamma-irradiation is generally associated with a fall in OER as large as that reported here. If this were so then the values for OER found in direct comparisons like these with ^{252}californium would have to be at least as low as those found with fast neutrons, if ^{252}californium is to provide a therapeutic advantage over existing gamma-ray sources.

Hall *et al.* [8] quote a range of values for the OER of ^{252}californium from 1·3 up to 1·9, which is the value in Table 1. In a detailed discussion of the present ^{252}californium data Nias *et al.* [3] concede the extreme possibility of fitting survival curves to their data which would reduce the OER to a value of 1·3. Even with this interpretation of their survival data, however, the gain factor would only amount to 1·09 with reference to the OER value found with ^{60}cobalt. It can thus be concluded, from the results obtained with this cell line at least, that beam therapy with fast neutrons at a conventional dose rate would provide a more useful innovation in clinical practice than interstitial ^{252}californium.

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REFERENCES

1. W. Duncan, Fast neutron therapy, past and present. *Acta Radiol. Suppl.* **313,** 11 (1972).
2. E. J. Hall, H. H. Rossi, A. M. Kellerer, L. Goodman and S. Marino, Radiobiological studies with monoenergetic neutrons. *Radiat. Res.* **54,** 431 (1973).
3. A. H. W. Nias, A. Howard, D. Greene and D. Major, The response of Chinese hamster (ovary) cells to protracted irradiation from ^{252}californium and ^{60}cobalt. *Brit. J. Radiol.* **46,** 991 (1973).
4. A. H. W. Nias, D. Greene and D. Major, Constancy of biological parameters in a 14 MeV field. *Int. J. Radiat. Biol.* **20,** 145 (1971).
5. C. W. Gilbert, Computer programmes for fitting Puck and probit survival curves. *Int. J. Radiat. Biol.* **16,** 323 (1969).
6. A. H. W. Nias, The clinical significance of cell survival curves. In *Biological and Clinical Basis of Tumor Radiosensitivity* (Edited by M. Friedman), Thomas, Springfield, Ill. (1974 In press).
7. E. J. Hall, Radiobiological measurements with 14 MeV neutrons. *Brit. J. Radiol.* **42,** 805 (1969).
8. E. J. Hall, L. A. Roizin-Towle and R. D. Colvett, RBE and OER determinations for radium and ^{252}Californium. *Radiology* **110,** 699 (1974).
9. M. Fox and A. H. W. Nias, The influence of recovery from sub-lethal damage on the response of cells to protracted irradiation at low dose-rate. In *Current Topics in Radiation Research,* (Edited by M. Ebert and A. Howard), Vol. VII, p. 71. North-Holland, Amsterdam (1970).
10. J. S. Bedford and E. J. Hall, Threshold hypoxia: its effect on the survival of mammalian cells irradiated at high and low dose-rates. *Brit. J. Radiol.* **39,** 896 (1966).

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Biological Effects of ^{252}Cf Neutrons*

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Abstract—*The biological effects of ^{252}Cf neutrons have been studied with cultured cells, and with intact tissues. Measurements available to date, of the relative biological effect (RBE) and of the oxygen enhancement ratio (OER), are summarized and discussed.*

INTRODUCTION

Biological effects of ^{252}Cf neutrons have been studied on the chromosomal level, with single cell cultures, and on intact tissues. Careful consideration of these data is required before embarking on clinical trials, since the relative lack of repair evidenced after ^{252}Cf irradiation produces a biological effect greater than that observed with X and γ-ray sources in general use. Additional problems are introduced since the composition of the mixed neutron-γ radiation field is a function of source incapsulation, depth in tissue, and extent of surrounding hydrogenous material. Also, experiments must be conducted at low dose rates used in therapy.

The effect of ^{252}Cf neutrons is generally measured relative to the effect produced by ^{226}Ra or ^{137}Cs γ-rays. In order to obtain meaningful comparisons, RBE values must be obtained for the neutron component alone. For survival curves, the slope of the mixed radiation survival curve can be related to the slope for neutron and γ components alone:

$$\frac{1}{D_0(n+\gamma)} = \frac{F_n}{D_0(n)} + \frac{F_\gamma}{D_0(\gamma)}$$

where F_n and F_γ represent the fraction of total absorbed dose delivered by neutrons and gamma rays respectively. In general, $D_0(\gamma)$ will not be known. If it is assumed that $D_0(\gamma)$ equals D_0 for the electromagnetic radiation relative to which the RBE is being calculated, the equation for RBE reduces to

$$\text{RBE} = \frac{D_{em} - D\gamma}{D_n}$$

Here, D_{em}, and $D_n + D\gamma$ are doses from standard electromagnetic radiation, and the mixed ^{252}Cf $(n+\gamma)$ field, respectively, which produce the same end point in the same irradiation time.

RELATIVE BIOLOGICAL EFFECT (RBE)

Table 1 presents the RBE data available to date [1]. Where necessary, the data has been manipulated to produce the RBE for neutrons alone, as outlined above. Data in Table 1 demonstrate an increase in RBE with decreasing dose rates. However, a comparison of results is complicated by the fact that RBE's can be quite sensitive to the γ standard dose rate used. This is caused by repair, as well as possible multiplicity changes. An examination of Table 1 shows that RBE's were not always obtained from the ratio of doses needed for equal effects and equal exposure times. Also, some cultured cell survival curve data indicate a lower RBE than that obtained with *in vivo* tissue systems. While this effect could be explained in the case of HeLa cells by their relatively low capacity for repair, this would not explain the RBE of 3·94 measured with hamster cells at 37° [1].

Evaluation of radiation effects on tissues have given higher RBE's. Withers *et al.*, found an RBE of 5 for mouse jejunal crypt cells, which he extrapolated to approximately 7 for ^{226}Ra at 35 rad/hr. Atkins *et al.*, reported an RBE of 6·6 for pig skin, relative to ^{137}Cs at 50 rad/hr.

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Table 1. *RBE of* ^{252}Cf

Biological system	Temp.	^{252}Cf total dose rate (rad/hr)	n/γ for ^{252}Cf radiations	γ Dose-rate of ^{137}Cs or ^{226}Ra standard	RBE (total dose)	RBE (neutrons only)	Author*
HeLa cells	37°C	16·0	1·7	31	2·89	3·95	Fairchild, Drew and Atkins, 1969
HeLa cells	30°C	20	1·95	60	2·15	2·74	Djordjevic, Anderson and Kim, 1973
HeLa cells	37°C	~13	1·7	37·3		4·38	Atkins, Fairchild and Drew, 1973
Hamster cells	37°C	1·27–5·21	1·23–1·59	11·7–52·5	10–5	16–7·7	Hall, Rossi and Roizin, 1971
Hamster cells	22°C	1·14–21·6	1·23–1·59	11·7–52·5	4·3 (^{226}Ra = 35·3 rad/hr)	6·7	Hall, 1972
Hamster cells	37°C	~13·5	1·33–1·7	37·3		3·94	Atkins, Fairchild and Drew 1973
Hamster cells	20°C	18	2·0	50	3·5	5·9	Hall, Roizin-Towle and Colvett, 1973
Hamster cells	22°C	15	1·17–2·0	100	3·5–6·7		Nias, Howard, Greene and Major, 1973
Hamster cells		167	2·09	79	1·72–2·28		Bushong, Prasad, Briney and Oliver, 1970
Chromosome Aberration Frequency		406–522	1·95	327	3·44–4·34		
P-388 Leukaemia cells	37°C	200–260	?	300–420	2·7–3·5		Berry, 1971
Vicia faba	22°C	16·02	1·32	46·9	6·5–5·3	10	Hall and Fairchild, 1970
Mouse jejunal Crypt cells	43·5	1·55		160	3·2	5	Withers, Oliver and Glenn, 1971
Pig skin		12–53 11·1	1·7	35–192 50		7·4–5·4 6·6	Atkins, Fairchild and Robertson, 1972
Mouse spleen colony		10·81	1·32	42·9	1·81	2·44	Carsten, 1972

*For references, see [1].

Effects on the hemopoietic system have been studied by Carsten. In a study on spleen colony-forming ability the RBE for californium neutrons was 2·44 compared to gamma irradiation. Dose rates were 10·81 rad/hr $(n+\gamma)$ from ^{252}Cf and 42·9 rad/hr gamma rays from ^{182}Ta. This is consistent with a somewhat lower RBE for hemopoietic tissues compared to other tissues for neutrons as shown by Hornsey and Field [2].

The higher RBE's such as those obtained with tissues (Fig. 1) [3] or with hamster cells at 22°C have been used in devising a dosage scheme for radiotherapy. Thus it has been suggested that 890 rad of ^{252}Cf neutrons in close to 7 days is equivalent to 6000 rad of radium γ-rays in 7 days, and that the dose rate used to compute the treatment time necessary to deliver the 890 rad should be: Cf neutron dose rate + 1/10 (Cf γ-ray dose rate) [4].

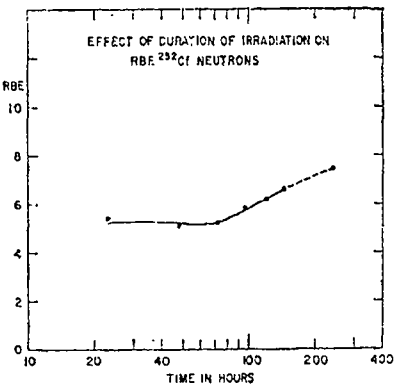


Fig. 1. *The relative biological effect of californium-252 neutrons, as measured by pig skin tolerance (compared to* ^{137}Cs *gamma rays or* ^{226}Ra *gamma rays for equal irradiation times).*

OXYGEN ENHANCEMENT RATIO (OER)

Another parameter of importance for the biological effect of ^{252}Cf neutrons is obtained by the ratio of doses which give the same effect in the absence and presence of oxygen (OER). OER measurements are of particular interest, since the rationale supporting neutron therapy pre-supposes that the efficacy with which neutrons act upon anoxic cells is greater than that of X- or γ- rays used in conventional radiotherapy.

Determinations of OER with ^{252}Cf neutrons have been consistent with other determinations performed with various sources of fast neutrons. Since OER is not a sensitive function of the ^{252}Cf gamma component, reports have in general been made on the basis of total dose $(n+\gamma)$. Measurements with ^{252}Cf have been made using growth of *Vicia faba* seedlings, chromosome aberrations in Chinese hamster cells and survival of P–388 leukemia cells, HeLa cells and hamster cells (Fig. 2). OER measurements (shown in Table 2) show quite good agreement, with values generally ranging from 1·4 to 2·0 A trend toward higher OERs is noted with higher dose rates, due presumably to an increasing contribution from the γ component. Clinical experience to date has been at the lower dose rates, such that the therapeutic gain factor

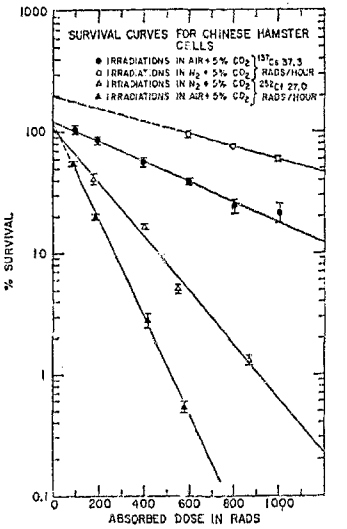


Fig. 2. Chinese hamster cell survival under oxygenation and anoxic conditions, after irradiation by ^{137}Cs gamma rays and ^{252}Cf neutrons. OER = 2·5 and 1·7, respectively [1].

$$\left(\text{TGF} = \frac{\text{OER of test radiation}}{\text{OER of standard radiation}}\right)$$

is (with the exception of the recent work by Nias [5]) significantly greater than 1.

Table 2. OER of ^{252}Cf

Biological system	Temp.	^{252}Cf total dose rate (rad/hr)	Cf OER	γ (^{137}Cs or ^{226}Ra) dose rate (rad/hr)	γ OER	Acute X-ray exposure OER	Author*
Vicia faba	22°C	16·02	1·66	46·9	2·02	2·7	Hall and Fairchild, 1970
Hamster cells Chromosome		60·4	1·75				Bushong, Prasad, Briney and Oliver, 1970
Aberration		167	1·76	79	2·32		Bushong, Prasad, Briney and Oliver, 1973
Frequency		406–522	1·81	327	2·29		
P-388 Leukaemia cells	37°C	200–260	2·0	300–420	2·6		Berry, 1971
HeLa cells	37°C	19·4	1·55	37·3	2·5	3·0	Drew, Fairchild and Atkins, 1972
HeLa cells	30°C	20	1·70	60·2	2·53	3·1	Djordjevic, Anderson and Kim, 1973
Hamster cells	22°C	16·5	1·4				Hall, 1972
Hamster cells	37°C	27·0	1·7	37·3	2·5		Atkins, Fairchild and Drew, 1973
Hamster cells	20°C	18	1·42	50	2·0	2·4	Hall, Roizin-Towle and Colvett, 1973
Hamster cells	22°C	15	2·18	100	1·42	3·2	Nias, Howard, Greene and Major, 1973

*For references, see [1].

Thus there is good biological evidence for an enhanced effect of ^{252}Cf neutrons on tumours because of a reduced necessity for the presence of oxygen during the period of irradiation.

HAZARDS

Biological considerations suggest caution in the use of ^{252}Cf neutrons in a hospital environment. The lack of recovery from cellular damage due to neutrons results in high values for relative biological effectiveness, particularly for low doses and low dose rates. This situation exists outside the treatment volume in interstitial implants with californium. Therefore it is not a simple matter to extrapolate from the experience with radium and other gamma sources insofar as effects on non-tumour tissue is concerned.

This is particularly true, in assessing the hazards to personnel who may be exposed to low doses over a long period of time. The RBE values for cancer induction (approximately 20), lens opacification (10–1000) and genetic damage (20) can be extremely high and should be kept in mind in the design of facilities for handling and storage.

REFERENCES

1. H. L. Atkins, R. G. Fairchild and R. M. Drew, Biological properties of ^{252}Cf. *Amer. J. Roentgenol.* **117,** 704 (1973).
2. S. Hornsey and S. B. Field, The RBE of cyclotron neutrons for effects on normal tissues. *Europ. J. Cancer* **10,** 231 (1974).
3. H. L. Atkins, R. G. Fairchild and J. S. Robertson, Dose-rate effects on RBE of californium and radium reactions of pig skin. *Radiology* **103,** 439 (1972).
4. E. J. Hall, Radiation dose-rate: a factor of importance in radiobiology and radiotherapy. *Brit. J. Radiol.* **45,** 81 (1972).
5. A. H. W. Nias, The response of Chinese hamster cells to ^{252}californium and 14 MeV neutrons. *Europ. J. Cancer* **10,** 301 (1974).

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Problems in Neutron Dosimetry*

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Abstract—*A summary of problems in neutron dosimetry is presented. Two categories are distinguished: those which pertain to physical measurements, and those which arise due to the nature of biological systems and the irradiation apparatus utilized, with special emphasis on the radiotherapy situation. The influence of radiation quality on almost all of these problems is discussed.*

INTRODUCTION

DURING the past seven months an International Neutron Dosimetry Intercomparison has been conducted at the Radiological Research Accelerator Facility at Brookhaven National Laboratory. Final results will be published elsewhere.

As host for this intercomparison, I had the good fortune to work with a number of scientists actively involved in performing neutron dosimetry, many of whom are considered expert in this field. I took this opportunity to impose upon their good nature by requesting that they briefly list their unofficial opinions as to what are the principle problems in neutron dosimetry and their recommendations apropos of these problems. The following summary is partly based on these comments.

PHYSICAL MEASUREMENT PROBLEMS

There are at present no standard neutron beams suitable for instrument calibration. It would obviously be desirable to establish such facilities at standards centres such as the Bureau International des poids et Mesures and the United States National Bureau of Standards. In addition it is important for dosimetry in general, to evaluate the types of instruments to be recommended with regard to accuracy, neutron energy response, neutron sensitivity, gamma ray sensitivity, size, convenience, etc. There is also a need to develop a good, directly responding, passive personnel neutron dosimeter for radiotherapy centres using either fast neutron beams or implants of neutron-emitting sources.

Of the fourteen scientists who participated in the international neutron dosimetry intercomparison, twelve chose to use ionization chambers. This indicates at least that ionization chambers are the most popular instruments. It can also be interpreted to mean that for a given amount of time, effort, and expense, these instruments are generally accepted as the most accurate in the majority of practical situations. Ionization chamber response is related to the energy absorbed in the gas by the quantity W, the average energy required to create an ion pair. There is a definite need to improve our knowledge of the *effective* value of W as a function of neutron energy for the various gases and gas mixtures used, especially for energies of less than about 100 keV.

Most materials used for so-called "tissue-equivalent" ionization chambers are deficient in oxygen and contain excess carbon compared to muscle. Thus, corrections are required for the higher energy absorption cross section of carbon and often also for resonances in carbon and oxygen. The significance of this problem depends on the neutron energy spectrum. Furthermore, the chambers usually are not strictly homogeneous, the gas being somewhat

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richer in oxygen than the wall, and this should also be taken into account by considering the spectrum.

As is well-known, neutrons are invariably accompanied by X and gamma rays so that the dosimetrist must decide which type of gamma ray dosimeter should be used in the mixed field. Energy-compensated Geiger-Müller gamma ray dosimeters are well suited to this task since their sensitivity for a neutron dose relative to that for the same gamma ray dose is less than 0·5% for neutron energies up to about 4 MeV, and probably does not exceed 3% at 15 MeV. Their application is limited, however, by their high sensitivity to photons, which requires increasingly significant corrections for counter dead-time as intensity increases.

Three of the participants in the neutron dosimetry intercomparison used GM dosimeters, five used thermoluminescent dosimeters or films, and nine used various so-called "neutron-insensitive" ionization chambers (some used more than one type of dosimeter). Although chambers can be used in quite intense photon fields, their sensitivities to neutrons are not well-known over a broad range of neutron energies, nor have the effects of chamber size and shape on this sensitivity been adequately studied. Similarly, for TLD and film better data are needed on their neutron sensitivity as a function of energy.

PROBLEMS RELATED TO CHARACTERISTICS OF BIOLOGICAL SYSTEMS

Increasing attention is being given to changes in neutron spectra produced at depth in biological systems by absorption and scattering. These changes depend on the size of the system, whether the beam is "monoenergetic" or has a distributed spectrum, and on the neutron energies present. It is generally accepted that spectra harden slightly at increasing depths. For some types of cells spectral modifications may be sufficient to cause significant changes in relative biological effectiveness. More measurements, both physical and biological, are needed.

There are also problems related to inhomogeneities in biological systems, especially in patients, with respect to the effects of density and atomic composition on the depth dose pattern and the dose absorbed in cells adjacent to interfaces. Again, these effects will depend on neutron energy spectrum. Although some interface dosimetry experiments have been performed with extrapolation chambers and by using monolayers of cultured cells adjacent to various materials, there is need for further work.

For neutron radiotherapy, there has yet to be resolved the influences of beam collimators and field size on neutron energy spectrum and gamma ray contamination. More measurements are also needed here.

Finally, there are questions concerning dose contributions from neutrons with energies between 1 and 100 keV, in particular the measurement of these doses, and evaluation of their biological significance.

It can be concluded that for physical measurement problems, spectral information is required to establish standard calibration fields, to evaluate W values and neutron sensitivities for photon dosimeters, and to apply corrections for lack of tissue equivalence and for ionization chamber inhomogeneities. For the evaluation of biological parameters such as RBE and OER, neutron spectra in tissue masses must also be known with respect to changes produced at depth and by tissue inhomogeneities, collimators, and field size. Consideration should also be given to the significance of epithermal and thermal neutrons. For all of these purposes the development is required of a simple and reliable neutron spectrometry system of *small* size to cover an adequately large energy range, say from 1 keV to 30 MeV.

SPECIFIC REMARKS

1. Absolute neutron dosimetry with tissue-equivalent ionization chambers requires two principal corrections; that for kerma in the chamber materials compared to tissue, which will decrease the value reported, and that for the effective value of W in the chamber gas, which will increase the value reported. Kerma corrections are generally applied with acceptable reliability, but dosimetrists do not yet have adequate data for making W corrections with comparable confidence.

2. Beam current is a useful monitor value for neutrons produced by bombardment of a beryllium target with deuterons. For neutrons produced by the $^3H(d,n)^4He$ reaction, beam current is less useful since target loading with tritium may not be uniform and will change with time. It can, however, be used to determine target replacement schedules.

3. Recognition should be given to the possibility of build-up of deuterium in targets since this may result in the generation of

relatively low energy neutrons which will degrade the spectrum.

4. Further problems to be decided upon for patient irradiations, are: (*a*) How many total dose and gamma ray dose monitors should be used, and where should they be located with respect to the target, collimator, and patient? (*b*) How, where, and when should spectra or beam quality be monitored?

5. Table 1 summarizes some characteristics of several dosimetry systems useful for fast neutrons. This information came from various sources, including the papers contributed for the discussion on dosimetry systems.

Table 1. *Characteristics of fast neutron dosimetry systems*

No.	Dosimeter	Accuracy of tissue dose determination %		Relative sensitivity (%)		Neutron enegy dependence
		Neutron	Gamma ray	Neutron	Gamma ray	relative to tissue
1.	Calorimeter, tissue equivalent or hydrogenous	~5 (p)	0·5–2 (p)	low	low	very low
2.	Ionization chamber, tissue equivalent	5–10 (p)	2–5 (p)	100	100	very low
3.	Scintillator, organic liquid	?	?	very high	very high	low
4.	Ferrous sulphate	10–15 (p)	2–6 (p)	moderate	moderate	moderate
5.	Thermoluminescent material	10–20 (p)	10–20 (p)	3–35	moderate	moderate
6.	Proportional counter, tissue equivalent	5–10 (m)	—	high	high	very low
7.	Fission chamber	5–10 (m)	—	moderate	very low	moderate
8.	Activation foils	5–10 (m)	—	low	very low	high
9.	Silicon diode	10–15 (m)	—	moderate	~0·1	moderate
10.	Precision long counter	5–30 (m)	—	high	very low	high
11.	Glass, Ag-activated phosphate	—	~5 (p)	0·5–4	moderate	—
12.	Geiger–Müller counter, energy compensated	—	~6 (p)	0·1–3	high	—
13.	Ionization chamber, non-hydrogeneous	—	3–20 (m)	2–30 ?	~100	moderate
14.	Photographic film	—	5–10 (p)	1–4	moderate	—

Note p: pure field.
m: mixed neutron and gamma ray field.

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Discussion on Dosimetry and Collimation of Fast-Neutron Beams*

A. PHYSICAL ASPECTS OF DOSIMETRY

H. ABEL, S. MATSCHKE and K. REGEL (Berlin-Buch, GDR): *Dosimetry of ^{9}Be(d, n)^{10}B neutrons for radiotherapeutic applications at the cyclotron in Rossendorf*

FOR THE determination of the neutron dose as well as of the gamma dose, spherical ionization chambers are used (o.d. 17 mm, wall thickness 1·5 mm). One of the chambers has a polyethylene wall and is flushed with ethylene, whereas the second chamber has a graphite wall and is flushed with CO_2. The sensitivities of the ionization chambers have been established by comparison with a calibrated gamma-ray dosimeter. We have estimated that the neutron dose is determined with an accuracy within ± 10%. Dose determinations with the activation method (S and Al) yield a deviation of −10%. Up to now, the variation of the neutron energy spectrum with depth in the phantom has not been determined experimentally.

Before patient irradiation the following tests are carried out:

(1) test of the correct function of the used dosimeters by means of calibrated gamma-ray source;
(2) check that only the Be target is bombarded with deuterons;
(3) test of the correspondence between the integrated target current, the measured value of a monitor chamber and the total dose in a tissue depth of 5 cm, measured with the polyethylene chamber.

The dose delivered to the patients during irradiation is controlled by the monitor chamber and the integrated target current.

P. R. ALMOND, A. R. SMITH (Houston, Texas, U.S.A.) and J. B. SMATHERS (College Station, Texas, U.S.A.): *The fast neutron beams from the TAMVEC cyclotron*†

FOR THE last twelve months patients from the University of Texas at Houston, M. D. Anderson Hospital and Tumor Institute, have been treated with the fast neutron beams produced by the Texas A & M Variable Energy Cyclotron (TAMVEC). Preliminary results were published [1] on the dosimetric properties of neutron beams produced by 16–60 MeV deuterons on beryllium. Basic dosimetry was carried out with tissue-equivalent ion chambers filled either with air or with tissue-equivalent gas.

Initially the patients were treated with neutrons produced by 16 MeV deuterons but due to the improvements in depth dose and skin sparing most of the treatments have been performed with the beam produced by 50 MeV deuterons. For the lower energy beam at 125 cm SSD the 50% total (neutron plus gamma) depth dose was found to vary from 7·7 cm for a 7 × 7 cm field to 9·4 cm for a 15 × 15 cm field. For the high energy beam at 140 cm SSD the 50% depth dose varied from 11·8 cm depth for a 7·5 × 7·5 cm field to 15·1 cm for a 22 × 22 cm field. These measurements were carried out in tissue-equivalent solution with a density of 1·065 g/cm^3. The depth of maximum dose was 2 mm for the beam from 16 MeV deuterons and 8 mm for the beam from 50 MeV deuterons.

The isodose curves have been obtained using decrement line methods and a computer controlled isodose plotter. Isodose distributions are calculated for any square or rectangular field

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*Full addresses of contributors to the discussion can be found in the list of participants to appear in Vol. 10, No. 6, pp. 393–398.

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from beam profile measurements at five depths for as few as three square fields and depth dose data from five field sizes. The isodose plotter measurements were taken to verify the accuracy of the computer generated isodose curves by the decrement line method and good agreement was found between the two. Curves were also obtained for wedge fields.

Silicon *p–i–n* diodes were found to be convenient and reliable integrating dosimeters for *in vivo* measurements with patients undergoing fast neutron therapy. These devices are almost completely insensitive to photons and thermal neutrons. Read-out does not alter the stored information. The diodes were calibrated directly on the treatment beam, using the change in forward voltage divided by the pre-irradiation forward voltage as the sensitive parameter. Annealing at 100°C was used to reduce and stabilize the forward voltage of previously exposed diodes prior to re-irradiation. The calibration to dV/V and the annealing permitted multiple re-use of the diodes with precision approaching 5% for doses from 50 to 350 rad. Doses below 50 rad were subject to greater uncertainties, with 50% precision at 4 rad. The small size and chemical inertness of the diodes enhance their utility as *in vivo* dosimeters. In animal and human trials, the diodes were successfully used to measure entrance and exit doses, dose at depth in body cavities, and dose distributions under special conditions.

REFERENCE

1. P. R. Almond, J. B. Smathers, G. D. Oliver, Jr., E. B. Hranitzky and K. Routt, Dosimetric properties of neutron beams produced by 16–60 MeV deuterons on beryllium: *Radiat. Res.* **54**, 24 (1973).

F. H. Attix, R. B. Theus, R. O. Bondelid (Washington, D.C., U.S.A.) and C. C. Rogers, (Richmond, Va., U.S.A.): *Neutron dosimetry for the MANTA facility at NRL*

The initial neutron dosimetry methods used at the MANTA (Middle Atlantic Neutron Therapy Association) facility have been described previously [1]. Since then two significant changes have been made as a result of later findings. These will be briefly summarized.

1. *Kerma correction for TE plastic*

As stated in the earlier paper [1] we formerly assumed that the A-150 type conducting tissue-equivalent plastic [2] employed for the construction of the ion chambers (EG & G, Inc., Goleta, Calif., U.S.A.) was perfectly tissue-equivalent with respect to the neutron kerma in our beam. We now estimate a correction for the difference in kerma between TE plastic (10·2% H, 76·1% C, 3·5% N, 10·2% O) and wet tissue (10·2% H, 12·3% C, 3·5% N, 74·0% O). We use the elemental kerma tabulations of Bach and Caswell [3], integrated over our neutron spectrum [4] up to the maximum energy (18 MeV) for which the kerma values are tabulated. We assume in the present approximation that the kerma ratio for TE plastic/wet tissue will not differ significantly above 18 MeV from its spectrally weighted average value below that energy (although we suspect it may actually rise to some extent as the cross section for carbon break-up reactions increases). The resulting kerma ratio K_{TE}/K_{tissue} is equal to 1·047 for our usual neutron beam from 35 MeV D^+ on Be. This ratio is applied to increase the neutron calibration factor B (ion current per neutron rad in tissue) employed with the TE plastic chambers, which results in a decrease in the measured neutron dose.

2. *Improved measurement of the gamma-ray dose*

Probably the main source of uncertainty in neutron dosimetry measurements is the determination of the gamma-ray dose component of the field. This stems from the unavailability of dosimeters which can measure gamma-ray dose without also responding in some degree to the neutron field. If the neutron/gamma-ray response ratio (B/A) can be established for the relevant neutron spectrum for two different dosimeters which differ strongly in their B/A values, their simultaneous measurements of the field will allow solution for the dose components D_n and D_γ.

We earlier tried to employ three simultaneous measurements; TE chambers and TLD's of LiF(TLD—700) and CaF_2:Mn, and employed a value of $(B/A)_{CaF_2}/(B/A)_{LiF} = 0{\cdot}57$ derived from results by Bewley and Parnell [5] to solve the simultaneous equations. We have since found that the B/A for CaF_2:Mn is unstable for reasons not yet clear, and that this method is therefore unreliable.

However, it is possible to employ Pb filtration in narrow-beam geometry to demonstrate that the gamma-ray dose component in our neutron beam is $<1\%$, and probably of the order of 0·1%. In that case the beam can be employed as a pure neutron beam to calibrate "gamma-ray" dosimeters (e.g. graphite, TLD) and thus obtain their relevant B values. In this way we have established that B/A for our graphite

chamber is about 0·30 and that for LiF is about 0·15–0·16. Further measurements are needed to improve the accuracy of these determinations. The Pb-filtration method offers a convenient and useful way of improving the accuracy of *n*–γ dosimetry.

REFERENCES

1. F. H. Attix, R. B. Theus, P. Shapiro, R. E. Surratt, A. E. Nash and S. G. Gorbics, Neutron beam dosimetry at the NRL cyclotron. *Phys. in Med Biol.* **16,** 497 (1973).
2. F. R. Shonka, J. E. Rose and G. Failla, Paper No. P/753, *Proc. Geneva Conf. Progress in Nuclear Energy*, Series XII, Vol. 1, p. 160 (1959).
3. R. L. Bach and R. S. Caswell, Energy transfer to matter by neutrons. *Radiat. Res.* **35,** 1 (1968)
4. R. B. Theus, R. O. Bondelid, F. H. Attix and C. C. Rogers, *Report of NRL Progress*, April (1973)
5. D. K. Bewley and C. J. Parnell, The fast neutron beam from the M.R.C. cyclotron. *Brit. J. Radiol.* **42,** 281 (1969).

H. Bichsel, J. Eenmaa, D. R. Schumacher, K. A. Weaver, D. L. Williams, P. Wootton and W. G. Wyckoff (Seattle, Wash., U.S.A.): *Fast neutron beam dosimetry: comparison of the ion chamber and proportional counter approaches**

The radiations used were neutrons generated by 21 MeV deuterons incident on a 1·5 mm beryllium target yielding a neutron beam of 8 MeV average energy and gamma rays from a ^{60}Co teletherapy apparatus.

Ionization chamber measurements were made in the gamma-ray and neutron beams using a three-terminal guarded ionization chamber (1 cm^3 nominal gas volume), constructed of tissue-equivalent conducting plastic (Shonka A150) and fitted with provisions for flowing selected gases through the chamber. Charge carried by the ions liberated in the gas by radiations were integrated by means of a solid state electrometer operating in the total feedback mode and a 5 1/2 digit digital electrometer to display the electrometer analogue output.

Gas flow rate was adjusted so that the gas pressures were equal to ambient atmospheric. Pressure changes through narrow orifices were remote from the ion chamber and followed by copper tubing. At these low flow rates and with this apparatus no evidence of mechanical generation of charge was demonstrable. Gas flow was continued until stable readings of ion charge were obtained for the gas investigated under irradiation. Flow times were always many hours. Gases used included tissue-equivalent gas (64·4% CH_4, 32·4% CO_2, 3·2% N_2), nitrogen argon, oxygen, carbon dioxide and methane. The collecting voltage was varied and the ion charge extrapolated to 100% collection using the 1/V plot method. The tissue-equivalent chamber was exposed in a ^{60}Co gamma-ray beam which had been calibrated using chambers bearing standards traceable to the National Bureau of Standards, Washington, the National Physical Laboratory, Teddington, and the Clinical Physics Center, Houston. The spread of calibrations referred to these standards is less than 1%. The absorbed dose in rad in tissue-equivalent plastic was calculated from the exposure using the data in ICRU report 10b [1], and corrected for wall attenuation. The volume of the chamber could then be calculated from a knowledge of W, stopping power ratio, wall to gas (S), and observed ionization (Q). The chamber was then exposed at a defined position in the neutron beam, in the presence of a monitor with various gases flowing through the chamber as in the photon case. The dose in rad in tissue-equivalent plastic per unit charge on the monitor were computed from the relationship

$$\frac{\text{neutron beam rad}}{Q_{\text{mon}}} = [^{60}\text{Co coulombs/rad}]^{-1} \cdot \frac{W_{pz}}{W_c} \cdot \frac{S_p}{S_c} \cdot \frac{Q_{TE}}{Q_{\text{mon}}}.$$

W_{pz} is the weighted average of proton and heavy ion components from proportional counter measurements. S_p was taken as that of protons weighted for the expected energy distribution consequent to the neutron beam used. Results for various gases gave a gas volume of 1·027 ± 0·017 cm^3 in the ^{60}Co gamma-ray experiment while the dose in rad per coulomb in the neutron beam experiment had a standard deviation of the order of 7·5%. Others have stated that the gas volume effect is negligible; the present results indicate a need for further work with regard to values of stopping powers and W to be adopted for the spectrum of secondary charged particles consequent to this neutron beam.

With the high voltage adjusted to give a multiplication gain of about 600, the pulses from a proportional counter with tissue-equivalent walls and filled with a tissue-equivalent gas at 113 torr at 20°C equivalent to a dia-

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meter $d_c = 2$ μm of biological material) were recorded as a pulse height spectrum. The counter was calibrated from the average pulse height produced by 5·5 MeV alpha particles from an internal source. The average alpha particle event size for the 2 μm diameter was calculated from the stopping power. The event distribution $N(Y)$ was calculated from the pulse height spectrum and the calibration. The dose distribution $D(Y)$ is then $Y \times N(Y)d_c$. The total dose is the integral of $D(Y)$ over Y. The components due to photons, high and low energy protons, alpha particles and heavy recoil nuclei can be identified and their contribution to the whole evaluated.

Agreement between proportional counter and chamber techniques is of the order of 6%. Several components of errors can be distinguished:

TE ionization chamber

$7{\cdot}09 \times 10^3 \frac{\text{rad}}{\text{Tgt coul}} \pm 7\%$

2% ^{60}Co gamma-ray calibration (1% statistics, 2% NBS calibration, 1% systematic error)

5% stopping power calculations

4% neutron beam instability

TE proportional counter

$7{\cdot}54 \times 10^3 \frac{\text{rad}}{\text{Tgt coul}} \pm 6\%$

1% energy calibration

5% stopping power calculations

4% neutron beam instability

In both techniques, the dose is measured for Shonka A150 tissue-equivalent plastic. The dose in tissue can be calculated from the neutron spectrum and kerma per unit fluence.

REFERENCE

1. ICRU Report 10b, *Physical Aspects of Irradiation*, NBS Handbook 85, Washington, D.C. (1964).

M. Bormann, R. Schmidt, R. Sittig (Hamburg Germany) and A. Hess (Hamburg–Eppendorf, Germany): *Energy distribution and depth doses of D–T neutrons in a tissue-equivalent phantom by activation threshold detectors*

After completion of the neutron-therapy equipment in Hamburg–Eppendorf, the first physical experiments will be concerned with the determination of the energy distribution and relative depth dose in a tissue-equivalent phantom by activation method with different foils.

The materials of the detectors have been chosen according to the following criteria:

(*a*) well-known excitation functions

(*b*) half-lives between 20 min and 20 hr

(*c*) radiation components that can be detected adequately by a NaI well scintillation counter (decay energy above the discrimination threshold of 75 keV)

(*d*) great abundance of the activated isotopes in natural targets

(*e*) different thresholds of the corresponding nuclear reactions between 0 and 15 MeV.

According to these aspects 12 detector materials could be selected. The thickness of the detector foils lies between 0·05 and 0·125 mm, dependent on the nuclear mass. The diameter is 22 mm, limited by the dimensions of the well of the scintillation counter. Square plates made of Shonka plastic will be used as tissue-equivalent phantom. The experimental procedure will be the following: ten foils of the same isotope will be activated in 10 different depths. This procedure will be repeated for each kind of foil. At each irradiation, a reference-foil will be activated simultaneously in order to correct for the fluctuation of the neutron yield during the different irradiations.

The gamma and β^+ activities of the foils will be measured with a NaI well scintillation counter. This counter detects all pulses above a discrimination threshold corresponding to 75 keV electron energy. Ten foils, simultaneously activated, can be counted successively by an automatic probe-changing equipment.

Determination of the detector efficiency, analysis of the activity curves and determination of the energy distributions of neutrons on the basis of the initial activities of the foils and well-known excitation functions will be performed by computer programs [1].

REFERENCE

1. M. Bormann, H. Neuert and W. Scobel, Tables and graphs of recommended (n, p), (n, d) and $(n, 2n)$ cross sections and excitation functions between 1 and 37 MeV. IAEA, Vienna. (To be published).

J. J Broerse and J. E. Broers-Challiss, (Rijswijk, The Netherlands): *Dosimetry of D–T neutrons for radiotherapeutic applications at Rijswijk*

Different types of tissue-equivalent (TE) ionization chambers have been employed for the determination of the total absorbed dose.

These include a spherical chamber (outer dia 23 mm, wall thickness 2 mm) and a disc-type chamber (outer dia 38 mm, total width 9·5 mm, wall thickness 2·4 mm) flushed with tissue-equivalent gas. Details concerning the tissue-equivalence of the materials employed can be found elsewhere [1]. The wall thickness of the chambers was selected in view of the maximum range of the recoil protons produced by the 15 MeV neutrons. For free-in-air kerma determinations of lower energy neutrons, small corrections must be applied in view of the attenuation in the chamber wall.

The gamma contamination of the neutron field has been determined by comparing the response of a tissue-equivalent chamber with that of an argon-flushed Magnox chamber of equal dimensions. The latter chamber has a small neutron sensitivity indicated by the coefficient k, which is dependent on the neutron energy and was estimated to be 0·14 for 15 MeV neutrons. In addition, a suitably shielded Geiger–Müller counter has been used as a gamma-ray dosimeter [2].

The sensitivity of the dosimeters is determined with a calibrated ^{137}Cs gamma source. On the basis of this calibration, the neutron sensitivities of the TE chambers are derived by applying W_a/W_β values of 1·04 for the 15 MeV neutrons [3]. It has recently been suggested that for lower energy neutrons, different W_a/W_β values will have to be applied [4]. In general, the total dose reported is determined by the TE chambers which are approximately equally sensitive to gamma and neutron radiation. Up to now, the variation of the neutron energy spectrum with depth in the phantom and distance from the beam axis has not been experimentally determined. Possible changes in the neutron energy spectrum at depth in a phantom will introduce additional inaccuracies due to the energy dependence of some of the parameters employed. It is estimated that the total dose at the site of the tumour is determined with an accuracy of ± 10% and the gamma dose with an accuracy of ± 15%. The accuracy which is required for the gamma dose determination is less than for 15 MeV neutrons, in view of the relatively small dose contribution of the gamma rays and the RBE value which at the relevant doses is smaller by a factor of at least 1·5 in comparison to that of 15 MeV neutrons [5].

During patient irradiations, the neutron beam is monitored using a disc-type transmission chamber which is inserted through the collimator and positioned in the beam line. In order to check the actual dose received by the tumour, sulphur activation detectors are attached to the skin of the patient at the exit position of the beam. Small condenser type ionization chambers are under construction which will be used in the future for determination of the dose received by the patient.

REFERENCES

1. J. J. Broerse, J. E. Broers-Challiss and T. Maruyama, Dosimetry of D–T neutrons for radiotherapeutic applications. In *Proc. 1st Symp. Neutron Dosimetry in Biology and Medicine*, Munich. (Edited by G. Burger, H. Schraube and H. G. Ebert). Commission of the European Communities, Luxembourg, p. 627 (1972).
2. J. E. Broers-Challiss, A. C. Engles, C. J. Bouts and J. J. Broerse, Depth–dose measurements of D–T neutrons in tissue-equivalent phantoms. In *Proc. IAEA Symp. Neutron Monitoring*, IAEA, Vienna, Vol. I, p. 210 (1973).
3. ICRU Report 10b, *Physical Aspects of Irradiation*, NBS Handbook 85, Washington (1964).
4. J. A. Dennis, Computed ionization and kerma values in neutron irradiated gases. *Phys. Med. Biol.* **18**, 379 (1973).
5. G. W. Barendsen and J. J. Broerse, Neutron dosimetry in biology and medicine. In *Proc. 1st Symp. Neutron Dosimetry in Biology and Medicine*, Munich (Edited by G. Burger, H. Schraube and H. G. Ebert). Commission of the European Communities, Luxembourg, p. 1 (1972).

M. R. Cleland and J. Ryan (Westbury, N.Y., U.S.A.): *Neutron dosimetry system for routine treatment control*

A dual-channel neutron dosimetry system has been developed for routine control of neutron therapy procedures at the University Hospital Hamburg–Eppendorf. The dose delivered to the patient is determined by integration circuits which switch off the accelerator when a preset dose has been delivered. Elapsed time meters can also be preset to provide back-up protection in case of dosimeter malfunction.

Each dosimetry channel consists of a tissue-equivalent ion chamber positioned near the neutron source inside the treatment head, a preamplifier located just outside the head and a controller-reader unit in the operator's console. The console unit displays four kinds of information: the dose rate, the time integral of the dose, the elapsed time and the current–time product of the accelerator beam. All displays are digital except for the dose rate.

The integrated dose is displayed on a five-digit electronic meter which reads to 9999·9

rad. The same number is recorded on a mechanical counter which is equipped with a comparator for preset dose registration. In case of a power failure during the treatment, the accumulated dose information will be preserved on the mechanical counter. The digital clock reads up to 99·9 min. It also has a comparator for the preset time function. The dose rate meter will be useful when adjusting the accelerator controls for maximum neutron intensity. The target current integrator displays the number of mA·min required to produce a dose of 20 rad. This number is corrected each time another 20 rad is recorded. In this way the target efficiency can be continuously monitored. The numbers for dose, treatment time and target-current time are also converted to standard 8421 BCD computer code and are available for automatic record storage.

The console unit can be checked conveniently by means of a test switch. This generates a standard signal which simulates a dose rate of 12·5 rad/min at 100 cm SSD. All internal circuits are checked by this signal. The power supply voltages are continuously monitored and any failure will switch off the neutron generator. The collection voltage supplied to the ion chambers is also monitored through separate cables which sense the voltage at the chambers.

The dosimetry system is interlocked with the neutron generator so that the operator must adhere to the following procedures:

1. Prepare the neutron generator for normal operation
2. Turn on the neutron dosimeter control units and return each preset comparator to zero
3. Reset the dose and time counters to zero
4. Set the comparators to the required dose and treatment time
5. Reset the interlock system of the neutron generator
6. Switch on the high voltage supply of the accelerator
7. Switch on the beam current of the accelerator.

Step 6 must be accomplished within one minute after step 3 or else steps 3 through 6 must be repeated. The timers begin to run at step 7. The neutron radiation will stop when either the preset dose or the preset time is obtained on either of the two identical units.

Absolute calibration of this system must be accomplished by comparison of the readings with another neutron dosimeter of known sensitivity located in the treatment field at 100 cm from the target.

Acknowledgement—The authors wish to acknowledge the valuable advice given by G. J. Parnell of Hammersmith Hospital, London and L. J. Goodman of Columbia University, New York, regarding the basic design concepts of this system.

D. Greene and D. Major (Manchester, U.K.): *Neutron dosimetry in Manchester*

The neutron dosimetry systems in use and our current estimates of the uncertainties on their results are summarized in Table 1, which

Table 1. Uncertainties in dose determinations employing different types of dosimeters and results of intercomparison

Type of dosimeter	15 MeV neutrons				Neutrons from ^{252}Cf			
	Uncertainty in dose			Inter-comparison	Uncertainty in dose			Inter-comparison
	$(n+\gamma)$	n	γ	$(n+\gamma)$	$(n+\gamma)$	n	γ	$(n+\gamma)$
Polythene–ethylene ionization chamber	±6%	—	—	1·00*	±6%	—	—	1·00*
Tissue-equipment ionization chamber	±6%	—	—	0·95 (0·96)	±6%	—	—	1·11 (1·10)
Carbon-CO_2 ionization chamber	—	±10%	±10%	—	—	±9%	±7%	—
Calorimeter†	±4%	—	—	1·00 (1·04)	—	—	—	—
LiF	—	—	±20%	—	—	—	±15%	—
$FeSO_4$	±6%	—	—	—	—	—	±7%	—

*Taken as reference.
†Dose in polythene; all other doses in soft tissue.

also gives the results of some intercomparisons between the various systems.

The polyethylene and tissue-equivalent chambers are calibrated with gamma rays and respond to the total neutron and gamma ray dose. When their readings are used to determine the dose in rad to soft tissue, the major factors contributing to the uncertainties are the uncertainties on the *Wn/We* ratios and the kerma ratios. The experimental intercomparisons in columns 5 and 9 of Table 1 show the two sets of results based on the same measurements but using alternative values for the *W* ratio. The results in plain figures are based on values of *Wn/We* for ethylene of 1·07 for both radiation quantities [1], while the bracketed figures are based on values of *Wn/We* for ethylene of 1·03 and 1·10 for 15 MeV fission spectrum neutrons, respectively [2]. The use of these alternatives only changes the results of the intercomparisons marginally. For the 15 MeV neutrons the result of the intercomparison is within the stated uncertainties. For the fission neutrons the doses given by the tissue equivalent chamber are significantly larger than those given by the polythene chamber. This result is probably due to the thin carbon layers which are required in the polythene chamber, which make a negligible contribution to the kerma entering the gas in the 15 MeV field, while for the lower energy neutrons a significant part of the kerma passing into the gas is generated in the carbon layer. This would reduce the sensitivity of the system, in accordance with the observed result.

The uncertainties on separate determinations of the neutron and gamma ray doses from the results given by tissue-equivalent and carbon ionization chambers, are greater than that on the total dose mainly due to difficulties in determining the sensitivity of the carbon chamber to neutrons. The uncertainties given for the measurements of ^{252}Cf neutrons are based on an analysis which takes account of the fact that the carbon chamber is not a Bragg–Gray cavity in the neutron field. The uncertainties on the results with 15 MeV neutrons are estimates based on first results from a similar analysis which is still in progress.

The calorimeter used was made of polythene. It was calibrated in a ^{60}Co gamma ray field and the results obtained in the neutron and gamma ray field are stated as dose in polythene. If this result is compared with the dose in polythene determined by the use of the polythene–ethylene ionization chamber, it can be interpreted as a determination of the *Wn/We* ratio for ethylene. Again the results of the intercomparison in plain figures corresponds to *Wn/We* of 1·07 while the bracketed figure uses the Dennis value of *Wn/We* for 15 MeV neutrons. It can be seen that the former figure gives the better agreement though both are within the stated uncertainty.

LiF dosimeter, in a nonhydrogenous container, has been used mainly for determining gamma ray doses in the mixed radiation fields. The large uncertainty on the results arises mainly from limited information about the direct response of the system to neutrons. Within these wide uncertainties the gamma ray doses determined with this system are in agreement with those from the two ionization chamber method at both radiation qualities.

The *G* values for the ferrous suplphate dosimeter are based on dosage measurements with the ionization chamber, consequently, meaningful figures were not obtained in the intercomparison studies.

REFERENCES

1. D. Greene, An ionization chamber for the dosimetry of 14 MeV neutron beams. *Phys. in Med. Biol.* **16,** 489 (1971).
2. J. A. Dennis, Computed ionization and kerma values in neutron irradiated gases. *Phys. in Med. Biol.* **18,** 379 (1973).

G. Hehn and G. Pfister (Stuttgart, Germany): *Neutron energy spectra in a phantom for the Hamburg and Heidelberg therapy units*

Since neutron energy spectra are not easy to measure, a computer simulation has been carried out to determine the local change of the energy spectrum in a standard man phantom (30 × 30 cm) for the therapy units in Hamburg [1] and Heidelberg [2]. Concerning the energy flux density of the fast neutrons at the beam entrance of the phantom, the spectrum is determined by the target and the collimator under consideration [3]. For the Hamburg therapy unit with a tritium target and a special collimator, there is a high peak of the spectrum at 14 MeV, a low minimum at 12 MeV and a gradual increase towards a second maximum in the upper keV region. This spectrum, which is typical for a new tritium target with iron collimator, changes rapidly with increasing depth in the phantom. As can be seen in Fig. 1 the dip at 12 MeV has disappeared so that the energy flux density gradually decreases with falling energy of the neutrons. As expected, the peak at 14 MeV is rapidly reduced.

In the case of cyclotron neutrons, as used in the Heidelberg unit with a deuterium gas target, the change of the spectrum with depth results in a reduction of the neutron component above 8 MeV and a relative increase below this energy. The results pertain to a target-to-skin distance of 75 cm and a field diameter of 10 cm in both cases. For smaller field diameters the spectrum changes are larger. The flux density values shown in Fig. 1 are given for a target emission of 6×10^{12} *n*/sec at the Hamburg unit [4]. The values shown for the Heidelberg unit are normalized to give the same tissue kerma rate at the beam entrance in the phantom allowing a better comparison. Maximum errors in the calculation of the flux are ±30% and for the kerma rate ±10%.

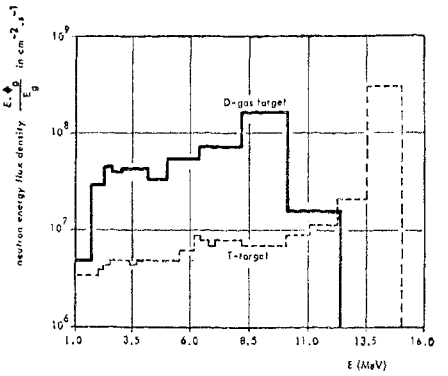


Fig. 1. Neutron energy flux density in a standard man phantom at a depth of 8·5 cm on the beam axis for a target-to-skin distance of 75 cm and a field size of 10 cm dia. Absolute values of the Hamburg therapy unit (tritium target) for a source of 6×10^{12} n/sec. *Relative values for the Heidelberg therapy unit (deuterium gas target) with normalization to the same tissue kerma rate at the skin (Hehn and Pfister).*

Spectrum changes with distance from the beam axis are smaller than the axial changes within the beam. There is a hardening effect near the edges of the beam which can be observed already at the beam entrance. The effect has a maximum at about 10 cm of depth and is lost after 20 cm. Beyond the beam edges there is a strong reduction of the high-energy components.

REFERENCES

1. H. D. Franke and A. Hess, Die Neutronen-Therapieeinheit (15 MeV) in der Radiologischen Universitätsklinik Hamburg-Eppendorf. *Kongress der Deutschen und Österreichischen Röntgengesellshaft, Wien* (1973).
2. W. J. Lorenz, F. Helms, K.-H. Höver, W. Maier-Borst and G. Wolber, Anwendungen des Heidelberger Kompaktzyklotrons. *Kongress der Deutschen und Österreichischen Radiologengesellschaft*, Wien (1973).
3. G. Hehn and R. Becker, Optimization of a collimator for 14 MeV neutrons. In *Proc. Ist Symp. Neutron Dosimetry in Biology and Medicine*, Munich (Edited by G. Burger, H. Schraube and H. G. Ebert). Commission of the European Communities, Luxembourg, p. 529 (1972).
4. G. Pfister, Untersuchungen zur optimalen Kollimation schneller Neutronen für die Neutronentherapie. IKE-Report (1973) (In preparation).

H. Kuhn (Frankfurt, Germany): *Dosimetry of fast neutrons for radiotherapy at the DKFZ cyclotron in Heidelberg*

A neutron beam monitoring system has been developed for the compact cyclotron at the Deutsches Krebsforschungszentrum (DKFZ) at Heidelberg. Since the cyclotron became operational this year and the neutron collimator assembly is under construction, the monitoring system and the calibration procedure have been tested at a 600 kV accelerator producing D–D and D–T neutrons.

1. *Beam monitoring system*

The monitoring system consists of a fission counter (^{238}U) wrapped with cadmium (thickness 0·1 cm) and a cylindrical tissue-equivalent ionization chamber (volume 11 cm^3). The electrical circuit for the fission chamber consists of a charge sensitive preamplifier, a discriminator, a main amplifier, and a digital display with the possibility to preset the desired number of counted pulses corresponding to a known neutron dose. Further, a ratemeter with recorder shows the variation of the neutron fluence rate.

The charge produced by the radiation in the monitor ionization chamber is determined with a digital current integrator with 3 separate input channels. In the channel provided for the monitor chamber it is possible to preset charge values so that a well defined dose can be given. A start-stop unit is able to switch the accelerator radiation, a time unit, and the operation of two other channels provided for two ionization chambers with different neutron response. It is possible to record the dose rate. Each input channel has 4 different charge sensitivities. The maximum charge sensitivity is $7{\cdot}00 \times 10^{-13}$ Cl/digit with an accuracy of 1% or less.

Since the fission chamber is only sensitive to fast neutrons with energies in excess of 1 MeV, a variation of the ratio of the reading of the the fission chamber to that of the monitor ionization chamber will indicate a variation of the gamma ray contribution and the amount of neutrons with energies less than 1 MeV within the beam. A determination of this ratio employing either deuterium or tritium targets showed only a variation within 1% for deuteron currents up to 2 mA at the 600 kV accelerator. Allowing a maximum dose increase of a few per cent the radiation is switched off automatically if one of both monitors fails.

2. *Calibration procedure*

The aim of the calibration procedure is to establish a relation between the displays of the monitors and the absorbed dose at the place of interest. The dose determinations have been performed with tissue-equivalent ionization chambers, polyethylene–ethylene chambers, and graphite–carbondioxide chambers. The different chambers have been used to estimate the total dose and the contribution from gamma radiation. The smallest ionization chambers which have been built, have a volume of about 2·6 cm^3, with an inner diameter of 1 cm and with a wall thickness of 0·05 cm which may be increased by caps. The radiation sensitivities of the ionization chambers have been determined experimentally with a calibrated ^{60}Co gamma ray source. For the calculations of the neutron sensitivities, knowledge of the ratios of the energy expenditure for gamma radiation and neutrons to produce an ion pair in the chamber gas is necessary. At the moment we use W_γ/W_n ratios of 0·96 for tissue-equivalent gas, 0·94 for ethylene and 0·91 for carbondioxide. These ratios are regarded to be energy independent although this is not strictly valid [1]. Further, the determination of the absorbed dose in tissue requires values of the ratios of the mass energy absorption coefficients of the corresponding materials for neutrons as well as for gamma radiation. These ratios have been calculated after values from Bach and Caswell [2], Ritts *et al.* [3], and Hubbel and Berger [4]. Details of the properties of the ionization chambers and about the method of dose determinations are given elsewhere [5].

For routine therapeutic applications of fast neutrons it is intended to check the monitors daily by measuring the neutron and gamma dose at a fixed distance at the centre of a cylindrical phantom with a height of 30 cm and a diameter of 20 cm.

REFERENCES

1. J. A. Dennis, Computed ionization and kerma values in neutron irradiated gases. *Phys. Med. Biol.* **18,** 379 (1973).
2. R. L. Bach and R. S. Caswell, Energy transfer to matter by neutrons. *Radiat. Res.* **35,** 1 (1968).
3. J. J. Ritts, M. Solomito and P. N. Stevens, Calculations of neutron fluence-to-kerma factors for the human body. *Nucl. Applic. Technol.* **7,** 89 (1969).
4. J. H. Hubbel and M. J. Berger, Photon attenuation and energy absorption coefficients. Tabulations and discussion. *Natl. Bureau of Standards Report* 8681, Washington (1966).
5. H. Kühn, Calibration and characteristics of ionization chambers for fast neutron irradiations. In *Proc. Symp. Dosimetry Techniques Applied to Agriculture, Industry, Biology and Medicine.* IAEA, Vienna (1973) (In press).

R. C. Lawson and D. Porter (Glasgow, U.K.)

Dosimetry for D–T neutrons at Glasgow

The total dose to wet tissue is measured using small cylindrical ionization chambers (outer diameter 10 mm, length 17 mm, wall thickness 2 mm). The wall materials are tissue-equivalent plastic, carbon loaded polythene, or normal polythene made conducting by a thin layer of graphite on the surface of the electrodes. Tissue-equivalent or ethylene gas is flowed continuously through the chambers.

An Orbitron ^{60}Co treatment unit is used to calibrate the chambers by the "in air exposure" method [1] against a calibrated Farmer–Baldwin ionization chamber. During the calibration all the chambers are fitted with appropriate build-up caps. The saturation characteristics, for neutrons and for gamma rays, are determined by plotting the reciprocal of the charge against $1/V^2$ for both positive and negative voltages.

For measurements in the neutron field, knowledge of the spectrum is used to assess the average neutron kerma values in the chamber materials and in wet tissue, and also to estimate the charged particle distribution in the walls and gas in order to calculate effective W values and stopping power ratios. The uncertainty in the total dose is 6%.

The neutron component of the dose is estimated by two different approaches. The primary method is to subtract from the total dose the gamma dose estimated from both measurements with ^7LiF TLD chips sandwiched in teflon and a neutron insensitive Geiger–Müller gamma ray dosimeter [2]. The

intrinsic neutron sensitivities of these devices are calculated from knowledge of the neutron spectrum and expressed as the ratio of the tissue dose in rad from ^{60}Co gamma rays relative to the tissue dose in rad for neutrons, required to give the same response. Typical sensitivities for spectra measured in a tissue-equivalent phantom irradiated by collimated 14·7 MeV neutrons [3] are 0·14 for ^{7}LiF [4, 5] and 0·025 for the Geiger–Müller dosimeter [2, 6].

In the second method, the neutron dose is measured directly, using calibrated ^{237}Np and ^{238}U pulse fission chambers which are insensitive to gamma rays. The ^{238}U chamber is surrounded by a thin cadmium sheath to remove all the thermal neutrons present which would otherwise react with the residual ^{235}U in the fissile material. Where the spectrum is known, the dose is deduced from the calculated tissue kerma and the mean neutron fluence measured by the chambers. Alternatively, for an unknown spectrum the combined response can be chosen to give an optimum match to the variation of tissue kerma with neutron energy. The dose, $K_n(FC)$, is then predicted using the counts $C(Np)$ and $C(U)$ from the fission chambers by the relation

$$K_n(FC) = aC(Np) + bC(U)$$

where a and b are the mixing coefficients. If the spectrum is known it can be incorporated in the optimization procedure. The neutron dose can be measured to $\pm 6\%$ by the fission chambers.

The influence of collimator shape and composition on the neutron spectra in collimated D–T neutron beams has been investigated and the variation in spectrum quality determined at depth in a phantom [3]. Only small changes in the spectrum shape were observed e.g. the percentage neutron dose due to neutrons above 12 MeV decreased from 73% at 2 cm depth to 63% at 17 cm.

REFERENCES

1. SCRAD, Protocol for the dosimetry of X- and gamma-ray beams with maximum energies between 0·6 and 50 MeV. *Phys. in med. Biol.* **16,** 379 (1971).
2. E. B. WAGNER and G. S. HURST, A Geiger–Müller gamma-ray dosimeter with low neutron sensitivity. *Hlth. Phys.* **5,** 20 (1961).
3. W. J. HANNAN, D. PORTER, R. C. LAWSON and R. RAILTON, Spectrum measurements in 15 MeV neutron therapy beams. *Phys. in med. Biol.* **18,** 808 (1973).
4. P. H. MCGINLEY, Response of LiF to fast neutrons. *Hlth. Phys.* **23,** 105 (1972).
5. C. L. WINGATE, E. TOCHILIN and N. GOLDSTEIN, Response of LiF to neutrons and charged particles. In *Proc. Int. Conf. Luminescence Dosimetry*, Stanford, p. 421 (1965).
6. L. J. GOODMAN, Neutron dosimetry at the Radiological Research Accelerator Facility. In *Proc. Int. Symp. Neutron Dosimetry in Biology Medicine*, Munich (Edited by G. BURGER, H. SCHRAUBE and H. G. EBERT). Commission of the European Communities, Luxembourg, p. 177 (1972).

N. PARMENTIER, M. CHEMTOB, A. RICOURT (Fontenay-aux-Roses, France), J. P. MEULDERS, Y. JONGEN, A. DEBAUCHE (Louvain-la-Neuve, Belgium) and A. WAMBERSIE (Louvain, Belgium): *Preliminary dose measurements in the fast neutron beam at the cyclotron of the University of Louvain*

THE TOTAL absorbed dose is determined with a disc-type ionization chamber constructed from tissue-equivalent (TE) plastic (outer diameter 38 mm, total width 10 mm, wall thickness 3 mm). A TE gas flows through the collecting volume. The wall thickness of the chamber is calculated for the maximum range of the recoil protons produced by the 14 MeV neutrons [1]. For energies beyond 14 MeV the charged particle equilibrium has been determined by increasing the wall thickness to 4·8 mm.

Two types of detectors are used for the determination of the gamma ray dose component:

(*a*) a disc-type ionization chamber with aluminium walls, of the same dimensions as the TE chamber, and flushed with argon gas. This chamber has a neutron sensitivity which is given by a factor k, depending on the neutron energy [2];

(*b*) thermoluminescent detector (TLD), enriched lithium-7 fluoride, the correction of the neutron sensitivity is described elsewhere [2].

All dosimeters used were calibrated by comparative measurements using a Baldwin Ionex chamber in a ^{60}Co gamma ray field. This secondary calibration instrument has been calibrated before in a well-known ^{60}Co gamma ray beam. For the TE chamber, a correction is applied for the difference in energy required to produce an ion pair for electrons and charged particles. Furthermore, the chamber response has been compared to kerma in tissue calculations by using a well-defined neutron beam from a Van de Graaff accelerator. A spherical TE proportional counter is used to determine the spectrum of dose vs LET.

The accuracy of total dose determination

with the TE chamber is about ±4%. For the gamma dose and the neutron dose, this accuracy is dependent on neutron energy. The following values of the accuracy could be estimated: neutron dose: at 14 MeV ± 7%, at 6·5 MeV ±5%; gamma dose: at 14 MeV ±18%, at 6·5 MeV ± 12%. For the higher energy neutrons, there is, at present, a lack of cross section data which are necessary for the *k* factor calculations.

The neutron beam is monitored with another disc shaped TE chamber. Measurements have shown that this monitor can be used with air replacing TE gas. In future applications, a greater disc chamber with air will be used as a monitor placed between the target and the collimator.

For the neutron beams produced by deuterons with energies of 16, 33 and 50 MeV, the percentage depth doses are given in Fig. 2, as determined in a TE liquid phantom. The spectrum of dose versus LET is given in Fig. 3.

REFERENCES

1. N. Parmentier, M. Chemtob and A. Ricourt, Dosimétrie de faisceaux de neûtrons pour les besoins de la radiobiologie. *Ann. Phys. Biol. Méd.* (To be published).
2. A. Debauche, P. Leleux, J. P. Meulders, C. Pirart, A. Wambersie, M. Winant, A. Bridier, M. Chemtob, P. Fache, R. Laguerre, V. D. Nguyen, N. Parmentier, A. Ricourt, R. Soulie and C. Veau, Caractérisation d'un faisceau de neutrons pour des études radiobiologiques. In *Proc. Coll. l'Effet de l'Exposition aux Neutrons sur la Fonction Cellulaire*, Munich. IAEA, Vienna (1973) (In press).

B. BIOLOGICAL ASPECTS OF DOSIMETRY

R. J. Berry (Oxford, U.K.) and D. K. Bewley (London, U.K.): *The importance of change in biological response with depth in tissue for collimated neutron beams—further experimental verification*

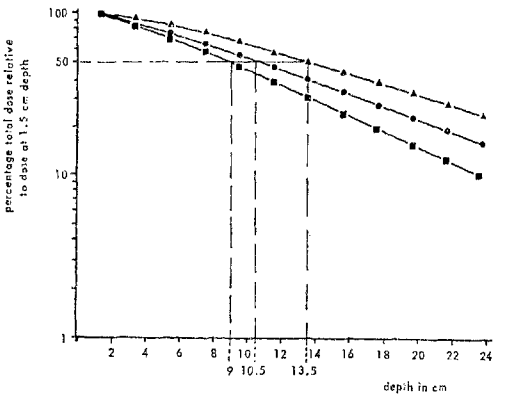


Fig. 2. Depth–dose curves in TE liquid phantom for neutron beams produced by 16 *MeV* (■), 33 *MeV* (●) *and* 50 *MeV* (▲) *deuterons on beryllium* (*Parmentier* et al.).

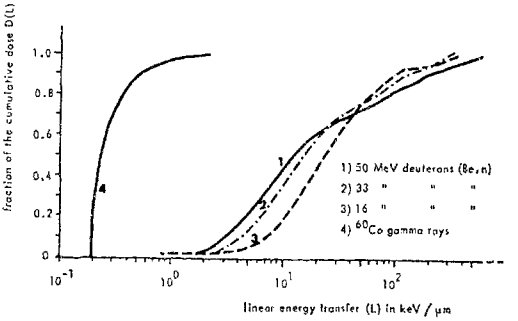


Fig. 3. The cumulative dose as a function of LET for ^{60}*Co gamma rays and three different neutron beams* (*Permentier* et al.).

EARLIER data obtained with murine P–388 leukaemia cells assayed *in vivo* after irradiation with collimated beams of fast neutrons of various average energies between 1 and 25 MeV, showed an unexpected decrease in hypoxic protection against cell killing by 6–25 MeV neutrons when the cells were irradiated 10 cm deep in a tissue-equivalent phantom, as compared to irradiation with no phantom [1]. The design of these experiments has, however, been criticised because the aerobic irradiations were carried out *in vitro*, whilst the hypoxic irradiations were carried out *in situ* in the tumour-bearing donor mice [2]. In all cases, however, the assay of cell reproductive survival was carried out *in vivo*. To attempt to answer this criticism, we have carried out two self-contained series of experiments using the collimated fast neutron beam from MRC cyclotron at Hammersmith Hospital (mean energy 7·5 MeV), in which both aerated and hypoxic exposures were delivered to P–388 leukaemia cells *in vitro*, prior to assaying survival of their reproductive capacity *in vivo*. The irradiation set-up was the same as was used by McNally and Bewley, who earlier found no significant change in oxygen enhancement ratio (OER) with depth in a phantom when using cells of another murine tumour as the biological assay system [3]. In the first set of experiments, using a field size of 10 × 10 cm at an SSD of 120 cm, exposures were carried out with no phantom present and at 8·7 cm depth in the phantom. In the second series, using a 16 × 16 cm field, exposures were given without the phantom and at 1·5, 15·5 and 23·3 cm depth, using the same dose rate in all cases. The cells were than assayed *in vivo* as in the earlier experiments. The results are shown in Fig. 4. Survival curves were fitted to the data by the method of Pike and Alper [4] and their parameters are summarized in Table 2. There was no significant change in the slope of the survival curve for aerated cells with depth so that a common curve could be fitted to the data for all depths. However, the slope of the survival curve for cells irradiated in nitrogen became steeper with increasing depth in the

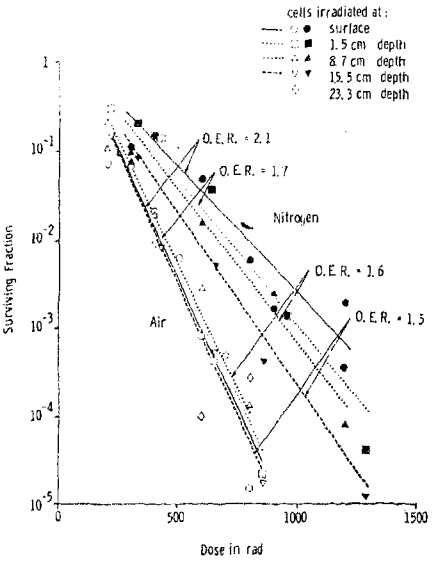


Fig. 4. Survival in vivo *of P–388 leukaemia cells irradiated* in vitro *with fast neutrons from the MRC cyclotron, Hammersmith Hospital. The "surface" data are for cells irradiated in the absence of the tissue-equivalent phantom (Berry and Bewley).*

Table 2. Survival curve parameters for P–388 leukaemia cells irradiated in vitro *with fast neutrons from the MRC cyclotron, Hammersmith Hospital, and assayed* in vivo

Depth in tissue-equivalent phantom (cm)	Extrapolation number (N)	D_0 (rad) air	D_0 (rad) nitrogen	OER
No phantom			146 (128–170)	1·9 (1·7–2·1)
1·5			127 (111–147)	1·6 (1·5–1·8)
8·7	2·0 (1·1–3·6)	78 (71–85)	122 (106–142)	1·5 (1·4–1·8)
15·5			109 (97–124)	1·4 (1·3–1·6)

Figures in brackets represent 95% confidence limits.

phantom, so that the OER was reduced significantly.

Although it is clearly not seen in some mammalian cell lines [5, 6] these results confirm that the P-388 leukaemia cell shows a significant change in its response to high energy neutrons at a depth in tissue-equivalent phantom material. At least one other mammalian cell type shows remarkable variation of response to the changes in neutron spectrum introduced by collimation [7]. Other types of both normal and malignant origin should be studied to determine whether this potentially useful increase in therapeutic gain factor at a depth in tissue can be expected to occur in the treatment of human tumours with external beams of fast neutrons.

REFERENCES

1. R. J. Berry, Hypoxic protection against fast neutrons of different energies—A review. *Europ. J. Cancer* **7,** 145 (1971).
2. 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).
3. 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).
4. M. C. Pike and T. Alper, A method for determining dose-modification factors. *Brit. J. Radiol.* **37,** 458 (1964).
5. A. H. W. Nias, D. Greene and D. Major, Constancy of biological parameters in a 14 MeV neutron field. *Int. J. Radiat. Biol.* **20,** 145 (1971).
6. R. Railton, R. C. Lawson, D. Porter and W. J. Hannan, Neutron spectrum dependence of r.b.e. and o.e.r. values. *Int. J. Radiat. Biol.* **23,** 509 (1973).
7. J. J. Broerse, A. C. Engels, P. Lelieveld, L. M. van Putten, W. Duncan, D. Greene, J. B. Massey, C. W. Gilbert, J. H. Hendry and A. Howard, The survival of colony-forming units in mouse bone marrow after *in vivo* irradiation with D–T neutrons, X- and gamma radiation. *Int. J. Radiat. Biol.* **19,** 101 (1971).

J. H. Hendry, D. Major and D. Greene (Manchester, U.K.): *The response of mouse intestine, irradiated at 10 cm depth in a water phantom with single and fractionated doses of 14 MeV neutrons*

The assay technique for intestinal damage, which measures crypt survival [1], has been used to determine the effectiveness of the collimated Manchester 14 MeV neutron beam in reducing crypt numbers to between 90% and 10% of normal, after single and fractionated doses delivered to mice in air and at 10 cm depth in a water phantom.

The "in air" RBE with respect to single gamma ray or X-ray doses decreases from about 2·0 (at 90% crypt number survival) to about 1·8 (at 10% survival). These values are intermediate between those reported for uncollimated 14 MeV neutron beams [2, 3] and the Hammersmith cyclotron [4]. The neutron beam is 8% less effective at 10 cm depth in water, in agreement with an earlier report using HeLa cells [5].

The iso-effect relationship (total dose vs number of daily fractions, up to 5) is linear on a log/log plot for both gamma rays and neutrons and the lines for increasing levels of damage are approximately parallel. The RBE values mentioned above for single doses increase to 3·2 and 2·5 respectively for 5 daily fractions, and for 4 fractions, the neutron beam is again 8% less effective at 10 cm depth in water. These RBE values for 5 fractions are higher than the values of about 2 reported for the uncollimated Rijswijk neutron beam [6], but this is to be expected in view of the different single dose RBE values.

The increase in RBE (from 1 to 5 fractions) at 90% survival (a factor 1·6) differs from the corresponding increase at 10% survival (a factor 1·4) by less than 10%.

REFERENCES

1. 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).
2. 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).
3. 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).
4. 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).
5. A. H. W. Nias, D. Greene and D. Major, Constancy of biological parameters in a 14 MeV field. *Int. J. Radiat. Biol.* **20,** 145 (1971).
6. J. J. Broerse and H. Roelse, Survival of intestinal crypt cells after fractionated exposure to X-rays and 15 MeV neutrons. *Int. J. Radiat. Biol.* **4,** 391 (1971).

R. MORRISON (London, U.K.): *Neutron dose distributions and RBE*

THE CHANGE in RBE of neutron irradiation with the size of the dose fraction may have some importance in treatment planning. From the graph published by Field and Hornsey [1] relating RBE of 7 MeV neutrons relative to 250 kV X-rays and the size of the dose fraction, it can be deduced that the value of the RBE changes particularly between dose fractions of 60 up to 200 rad. This is within the range used in therapy and may affect the calculations of depth doses and dosage in the penumbra.

In Table 3, the RBE values for dose fractions at different depths are given for an incident dose of 200 rad of neutrons. It will be seen that if the neutron dose is related to a dose of 250 kV X-rays, an equivalent depth dose is considerably greater than is suggested by the isodose curves, e.g. at the 50% isodose level the equivalent X-ray dose would be 63% of the incident dose—this is similar to the depth dose obtained from a telecobalt unit at an SSD of 75 cm.

If fractions of only 100 neutron rad are given at each session, the increase is even greater. At the 70% level the equivalent 250 kV X-ray dosage would be 86%. This is the same depth dose that would be received from a 6 MeV linear accelerator. By using smaller fractions it is possible to obtain higher equivalent depth doses.

These findings may have some importance in treatment field planning and in field arrangement. It may be better for example to use opposing fields than nonopposing or angled fields because of the relatively greater damage to the areas receiving small fractions.

REFERENCE

1. 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).

P. TODD (University Park, Penna., U.S.A.): *Preliminary biological evaluation of neutron collimation at the Naval Research Laboratory cyclotron*

HUMAN kidney T–1 cell survival was determined as a function of distance from the beam centre line by irradiating attached cells in plastic bottles as in earlier work [1]. The bottles were placed at various depths in the tissue-equivalent liquid phantom. A preliminary iso-survival profile using a 10 × 10 cm collimator [2] is shown in Fig. 5, and the equivalent gamma ray dose is also given. Additional experiments indicated that off-beam cell lethality was attributable to neutrons.

REFERENCES

1. P. TODD, C. B. SCHROY, K. G. VOSBURGH and W. SCHIMMERLING, Spatial distribution of biological effect in a 3·9 GeV nitrogen ion beam. *Science* **174,** 1127 (1971).
2. F. H. ATTIX, R. B. THEUS, R. O. BONDELID and C. C. ROGERS, Neutron dosimetry for the MANTA facility at NRL. *Europ. J. Cancer* **10,** 314 (1974).

C. COLLIMATION OF NEUTRON BEAMS

G. BREYNAT, P. BORY, S. MARCIE, G. N. LALANNE and A. COSTA (Grenoble, France): *Collimation of a 14 MeV neutron beam*

Table 3. Depth dose data for 7 MeV neutrons and equivalent X-ray data

	7 MeV neutrons field size: 9·5 × 9·5 cm			Equivalent dose 250 kV X-rays	
	Depth dose (%)	Dose fraction (rad)	RBE	Dose (rad)	Depth dose (%)
Dose fraction	100	200	2·7	540	100
of neutrons:	80	160	2·9	464	86
200 rad	60	120	3·2	384	71
	40	80	3·76	301	58
Dose fraction	100	100	3·4	340	100
of neutrons:	90	90	3·54	319	94
100 rad	80	80	3·76	301	89
	70	70	4·16	291	86

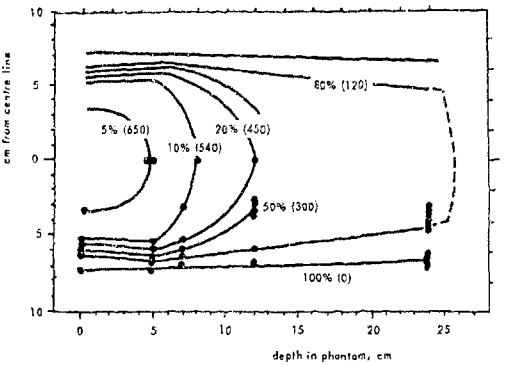


Fig. 5. Iso-survival profile in tissue-equivalent fluid for cultured human kidney T-1 cells irradiated in the 10 × 10 cm neutron beam of the Naval Research Laboratory cyclotron. A 183 rad neutron dose was deposited on the centre line at a depth of 12·3 cm. Iso-effect lines are indicated for different percentages of survival, the values in parentheses give the equivalent gamma ray doses (Todd).

THE COLLIMATION of 14 MeV neutrons has been studied experimentally for a maximum collimator thickness of 50 cm, which will allow a source-to-skin distance (SSD) of 60 cm. Three different types of collimators have been employed, consisting of steel, steel plus polythene and steel plus tungsten for field openings of 5 × 5, 5 × 12, 12 × 12 and 14 × 14 cm.

Measurements of the neutron flux density were performed with various threshold detectors: copper in cadmium (neutron energy threshold 11 MeV), phosphor in cadmium-aluminium (neutron energy threshold 2 MeV) and dysprosium (thermal neutrons). A cylindrical polythene-ethylene ionization chamber (volume 0·2 cm^3), constructed according to the design of Greene [1], has been employed for the determination of the absorbed dose due to neutrons and gamma radiation. An estimation of the gamma contamination of the neutron field has been derived from measurements with photographic film, LiF dosimeters, CaF_2 dosimeters (MBLE) and Victoreen chambers with relative neutron sensitivities of 0·02 ± 0·01, 0·25 ± 0·10, 0·10 ± 0·02 and 0·8 ± 0·1, respectively. The CaF_2 thermoluminescent

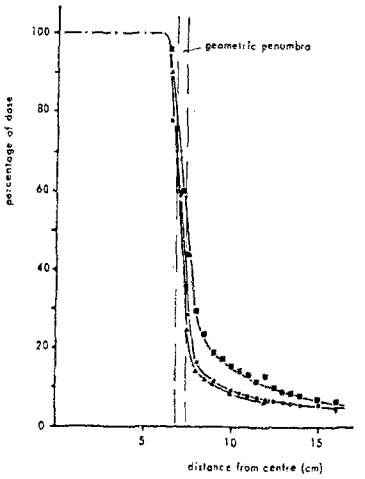


Fig. 6. Beam profile curves measured free-in-air (SSD 64 cm, field size 14 × 14 cm) for three different types of collimators, ●: steel, ■: polyethylene and ▲: tungsten (Breynat et al.).

Table 4. Relative flux densities of neutrons [in n/(cm² sec)] in subsequent energy intervals and dose rate of contaminating gamma radiation for neutron beams employing different types of collimators (SSD 64 cm, field size 12 × 12 cm, total yield 10^{11} n/sec)

Range of neutron energies (E_n)	Type of collimator		
	Steel	Steel plus polythene	Steel plus tungsten
$E_n > 11$ MeV	$2 \cdot 10^6$	$2 \cdot 10^6$	$2 \cdot 10^6$
2 MeV $< E_n <$ 11 MeV	$5 \cdot 10^4$	$5 \cdot 10^5$	10^5
Intermediate neutrons*	10^2	10^2	$2 \cdot 10^2$
Thermal neutrons	$4 \cdot 10^4$	$4 \cdot 10^4$	$2 \cdot 10^4$
Gamma radiation†	0·9	3·3	1·7

*The neutron flux densities for intermediate neutrons were estimated from calculations.

†The dose rate due to gamma radiation is expressed in rad/hr.

dosimeters have generally been used for the determination of the gamma contamination.

Relative values of the total absorbed dose have been measured free-in-air as a function of the lateral distance from the beam axis. The beam profile curves presented in Fig. 6 for the different collimators show that the collimator consisting of steel and tungsten provides the better definition of the beam in air. The composition of the collimated neutron beam, as measured free-in-air at an SSD of 64 cm and with a 12 × 12 cm field, is given in Table 4 for a total yield of 10^{11} *n*/sec from the 14 MeV neutron generator. The characteristics of the collimated neutron beams have also been studied for smaller fields of 5 × 5 and 5 × 12 cm.

Depth-dose measurements have been carried out with a rectangular polyethylene phantom (dimensions 25 × 25 × 100 cm). The isodose curves, determined with the polythene–ethylene ionization chamber, for two different collimators are presented in Fig. 7 for an SSD of 64 cm and a 14 × 14 cm field. Changes in field size will inevitably modify the depth–dose distributions in the patient.

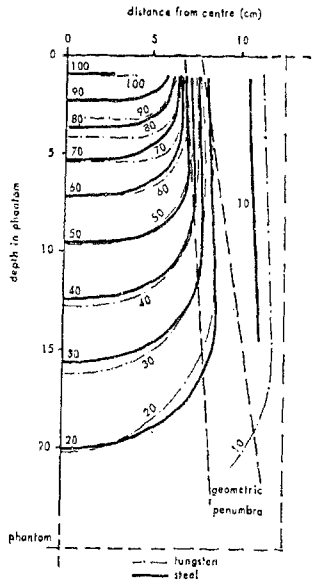


Fig. 7. Isodose curves measured in a polyethylene phantom for two different types of collimators (SSD 64 cm, field size 14 × 14 cm) (Breynat et al.).

REFERENCE

1. D. Greene, An ionization chamber for the dosimetry of 14 MeV neutron beams. *Phys. in med. Biol.* **16,** 489 (1971).

G. Burger, F. Grünauer, H. Schraube, (Neuherberg, Germany), W. J. Lorenz, K. -H. Höver, G. Wolber and G. Hartmann (Heidelberg, Germany): *Calculation of depth dose and beam profile for the fast neutron beam produced by the Heidelberg cyclotron*

A Device for neutron shielding and collimation of the neutron therapy facility at the Institut für Nuklearmedizin at Heidelberg was developed. The investigations were carried out by calculating the neutron and gamma radiation transport through the assembly and in a phantom.

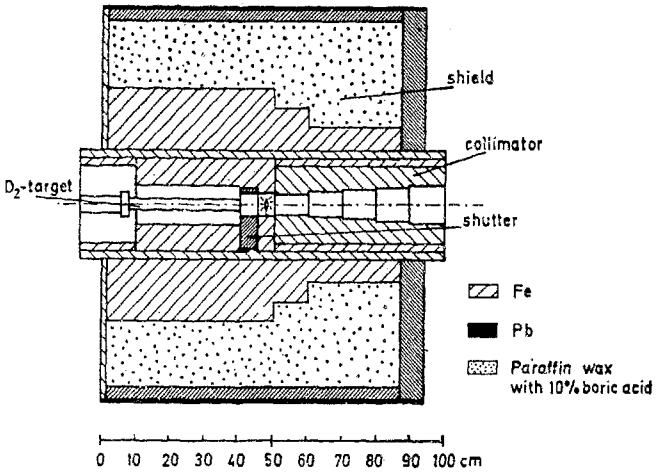


Fig. 8. Schematic drawing of collimator and shield (Burger et al.).

The shield essentially consists of an inner iron core including the iron collimator itself, an outer paraffin wax layer with 10% content of boric acid and a lead shield for additional removal of gamma radiation (Fig. 8).

With the standard patient position the distance from the target entrance window to the skin is 95 cm. The collimator itself is interchangeable. Its outer dimensions are 50 cm length and 20 cm dia. Three collimators are being built providing field sizes of 6×8 cm, 8×10 cm and 10×15 cm.

A lead shutter of 5 cm thickness provides sufficient gamma shielding from the highly activated inner part of the device for the patient and staff during positioning. For the total arrangement including the source data of a D_2 gas target [1] the isodose curves in a cylindrical phantom have been calculated. The calculations have been performed on the basis of the two dimensional multigroup—transport code DOT 2 [2], employing a total number of 21 energy groups. Cross section data and the fluence-to-kerma function were taken from [3] and [4], respectively. In order to show the influence of the shield and the collimator, the calculations have also been performed for an ideal aperture-beam under the same geometrical conditions. Figure 9 shows the beam profile curves for doses normalized at 10 cm axial depth.

It can be concluded that:

(*a*) the axial depth dose is not essentially influenced by the stray and leakage neutrons from the shield

(*b*) these neutrons play a significant role only outside the beam and mainly near the phantom surface

(*c*) the shield causes a relatively high increase of the gamma ray dose outside the beam.

REFERENCES

1. H. Schraube, H. Johlige and A. Morhart, Deuterium targets as fast neutron sources on a compact cyclotron. In *Proc. 1st Neutron Dosimetry in Biology and Medicine*, Munich (Edited by G. Burger, H. Schraube and H. G. Ebert). Commission of the European Communities, Luxembourg, p. 757 (1972).
2. F. R. Mynatt, F. J. Muckenthaler and and P. N. Stevens, *Development of Two-Dimensional Discrete Ordinates Transport Theory for Radiation for Shielding*, CTC-INF-952, AD-692168 (1969).
3. H. Fars, 112 group coupled neutron and gamma-ray transport code cross section data. ORNL Report TM-3049.
4. J. J. Ritts, M. Solomito and P. N. Stevens, Calculation of neutron fluence-to-kerma factor for the human body. *Nucl. Appl. Technol.* **7**, 89 (1969).

E. P. Cytacki and C. A. Kelsey (Madison, Wisconsin, U.S.A.): *Contribution of patient scatter to 15 MeV neutron beam penumbra*

The amount of scatter produced within a patient has been experimentally determined using a technique which eliminates contributions of room background and collimator transmission. The associated particle (AP) method of detection was used. In this technique a coincidence between the outgoing neutron scattered in a patient phantom and the alpha particle associated with that particular neutron

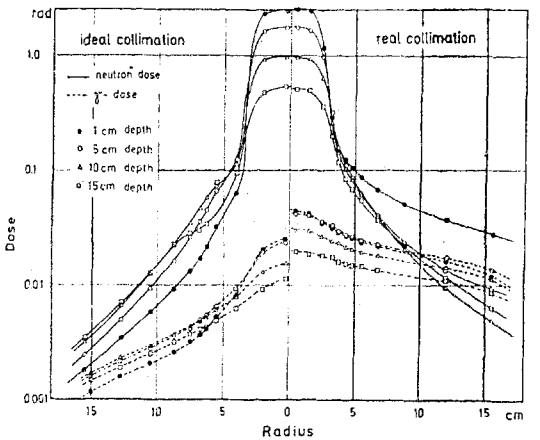


Fig. 9. Dose profiles in various depths of phantom for ideal and real collimation (Burger et al.*).*

is required before a count is recorded. The associated alpha particle was detected by a 100 μm silicon surface barrier detector. Collimation of the associated alpha particles effectively collimated the neutron beam. A 1·0 micron nickel foil covered the surface barrier detector to prevent the elastically scattered deuterons from reaching the detector. The neutrons were detected in a NE213 liquid scintillator detector which employed a pulse shape gamma discrimination circuit to eliminate pulses arising from gamma rays. A neutron pulse from the liquid scintillator was counted only if it came in coincidence (70 μsec window) with an alpha particle count.

The shape of the neutron beam was measured in air and at depths of 5, 10, and 15 cm inside a water phantom located at an SSD of 125 cm from the neutron source. Field sizes of 5 × 5 cm, 11 × 11 cm and 15 × 15 cm were investigated. All measurements were normalized to an equal number of alpha particle pulses. In a separate experiment the attenuation coefficient of water for 15 MeV neutrons was measured as 0·103 ± 0·01 cm^{-1} using the AP beam in a good geometry experiment. Using this value of the attenuation coefficient, the known depth in water, and the measured ratio of neutron counts per alpha count in air, the number of primary neutrons per alpha at a particular position in the water phantom could be calculated. This value was subtracted from the measured value to give the number of scattered neutrons at each position. The data were normalized to the central point of an 11 × 11 cm field at a depth of 5 cm in water. The values given in Table 5 are thus percentages of the maximum dose for the 11 × 11 cm field at an SSD of 125 cm. All measurements have an estimated overall uncertainty of ± 5% with an uncertainty in the position of the neutron detector of ± 2 mm.

Table 5. *Dose in penumbra region due to neutron scatter within a patient as percentage of dose maximum*

Depth in phantom (cm)	Field size 5 × 5 cm		11 × 11 cm		15 × 15 cm	
	Distance from edge* of beam 4 cm	7 cm	4 cm	7 cm	4 cm	7 cm
5	2·4	1·0	4·8	1·9	4·8	2·3
10	3·7	1·5	5·5	2·6	7·4	3·4
15	3·1	1·4	5·5	2·8	7·9	4·1

*Edge is taken at 50% isodose line.

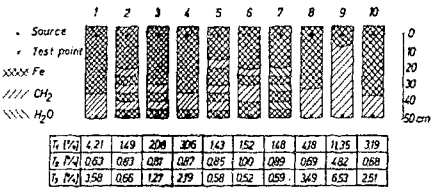
F. Grünauer and G. Burger (Neuherberg, Germany): *Some aspects in neutron shielding and collimation*

From the numerous shielding and collimation problems two small, rather independent aspects are discussed, namely:

1. which materials and which arrangements (bulky or multilayered) will yield optimum dose removal per given total shield thickness for 15 MeV neutrons;

2. what is the maximum radioactivity induced in the iron collimators during normal treatment runs and which problems arise with respect to their handling and shielding.

1. Radiation transmission has been calculated for a number of simple arrangements, using the one-dimensional SN-Code ANISN and the data library DLC-9 from RSIC-Oak Ridge [1]. An isotropic 15 MeV neutron source is positioned at the centre of a sphere with a total



	1	2	3	4	5	6	7	8	9	10
T_1 [%]	4.21	1.49	2.08	3.06	1.43	1.52	1.48	4.18	11.35	3.19
T_2 [%]	0.63	0.63	0.87	0.87	0.85	1.00	0.89	0.89	4.82	0.68
T_3 [%]	3.58	0.86	1.27	2.19	0.58	0.52	0.59	3.49	6.53	2.51

T_1: relative total dose T_2: relative neutron dose
T_3: relative γ-dose

Fig. 10. *Efficiency of shield lamination for a* 15 *MeV neutron source* (*Grünauer and Burger*).

radius of 50 cm. The sphere is divided into 20 layers, each 2·5 cm thick, which either consist of iron, polyethylene or water. For each chosen arrangement the neutron, gamma and total leakage doses have been calculated and normalized to the neutron doses at the same distance without shielding. The resulting dose transmissions are summarized in Fig. 10. The results show the following:

(*a*) Optimum neutron dose removal is achieved by a 35 cm iron core, followed by a 15 cm polyethylene layer;

(*b*) Optimum total dose removal is achieved by a multilayered arrangement. (Cases 1–6 have essentially been checked experimentally by Maruyama and Bouts [2]. The qualitative agreement is good)

The conclusion is that a multilayer arrangement of iron and hydrogeneous material in therapy-shieldings of 15 MeV neutrons is of little value. The critical neutron leakage dose (with a relatively high RBE) is not removed optimally. On the other hand sufficient gamma

dose removal should be achievable by mixing boric acid into the hydrogeneous material and using an additional outer lead shielding.

In order to calculate the activation of the shielding near the source and specifically of the interchangeable collimators of the Heidelberg Neutron Therapy Facility 3, the following daily treatment scheme was supposed: irradiations will only be performed from Monday to Friday, with 16 sessions per day from 9.00 a.m. to 4.45 p.m. Each irradiation will last 15 min followed by a 15-min interruption.

For the source and shielding device under consideration 3, first the spatial distribution of the saturation activation of ^{56}Mn, which has proved to be the only important nuclide, has been calculated. (The neutron source output was assumed to produce at 10 cm depth a dose rate of 30 rad/min). From the gamma source distribution the dose rates have been calculated again by the radiation transport code DOT. Fig. 11 shows the dose rates at 10 cm distance from the shield and around the isolated collimator. From these data and the temporary activation curve of ^{56}Mn for the mentioned irradiation scheme the true dose rates can easily be calculated for each time. As the ^{56}Mn activation (half-life time 2·57 hr) never reaches 50% of its saturation value during day time and vanishes practically completely during night time, it can be concluded that no serious activation problems have to be expected with the use of iron collimators.

REFERENCES

1. H. Fars, 122 group coupled neutron and gamma-ray transport code cross section data. ORNL Report TM-3049.
2. T. Maruyama and C. J. Bouts, Attenuation of 15 MeV neutrons in multilayer shields composed of steel, polyethylene and borated materials. *Phys. in med. Biol.* **17,** 420 (1972).
3. W. J. Lorenz, K.-H. Höver, H. Kuttig, G. Wolber, G. Burger, F. Grünauer and H. Schraube, Neutron therapy application at the Heidelberg cyclotron facility. *Europ. J. Cancer* **10,** 262 (1974).

K. M. Jones and C. A. Kelsey (Madison, Wisconsin, U.S.A.): *Experimental studies in the collimation of* 14·8 *MeV neutrons*

Measurements have been made using the associated particle (AP) method on neutrons scattered from the face of a 52·5 cm tapered steel collimator. The quantities measured include (1) the distribution of the scattered neutrons in the field and in the penumbra; (2) the shape of the penumbra due to neutrons transmitted through the edge of the collimator; (3) the distribution of points at which the scattered neutrons entered the collimator; (4) the distribution of sites from which they scatter; (5) the energy of the scattered neutrons.

The neutron source was an Activatron 211 neutron generator from Accelerators Incorporated. The deuterons from the accelerator were collimated to a 1 mm dia beam (2–10 μA) before striking the slowly rotating 4 Ci tritium target. The characteristics of the solid state detector employed for the detection of the alpha particle, and of the neutron detector are described elsewhere in this discussion session [1]. All measurements were made along a line 125 cm from the source and 58·8 cm from the end of the collimators.

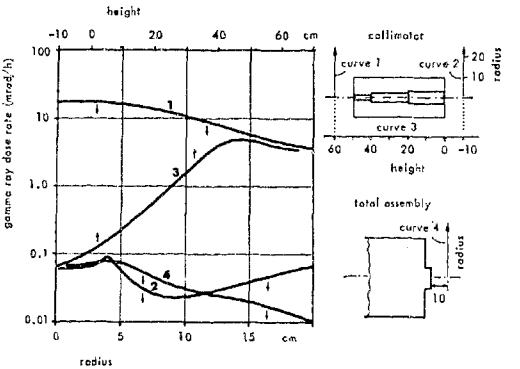


Fig. 11. *Gamma ray dose rate after irradiation in the surrounding of the collimator, due to saturation of* ^{56}*Mn concentration for a neutron source output producing a dose rate of* 30 *rad/min at* 10 *cm depth (Grünauer and Burger).*

The AP neutron beam was directed at only half of a 2 piece, single plane collimator. The penumbra on one side of the field consisted entirely of neutrons transmitted through the edge of the collimator while that on the other side consisted entirely of scattered neutrons. The scatter from a 4 piece, 2 plane collimator was calculated on the basis of the data obtained from the single plane collimator.

The results of the measurements listed above are summarized as follows.

(1) The total scatter by a 2 plane collimator into a 20×20 cm field is equal to $(6{\cdot}88 \pm 0{\cdot}23)\%$ of the direct flux and is constant across the field. At the edge of the field it drops to $(5{\cdot}08 \pm 0{\cdot}18)\%$ as one quarter of the collimator is removed as a potential scatterer. From the edge of the field, there is an almost straight line decrease to $(1{\cdot}68 \pm 0{\cdot}08)\%$ at 9 cm from the edge of the field. The percentage of scatter is proportional to the area of the collimator face being irradiated.

(2) When plotted, the neutron flux transmitted through the edge of the collimator gives a sigmoid shaped curve passing through a value of 2% of the direct flux 3 cm from the edge of the field.

(3) By directing the AP neutron beam at various segments of the collimator entry points for neutrons which will be scattered was determined. The collimator face was mathematically divided into 21 segments, starting at the source and the curve given by

$$Y_N = \sum_{i=1}^{N} \frac{\sigma_i \cdot \Delta\theta_i}{R_i}$$

was calculated. σ_i is the cross section for elastic scattering of neutrons on iron from the i^{th} segment to the detector, $\Delta\theta_i$ is the angle subtended by the i^{th} segment at the target, and R_i is the distance from the segment to the detector. The experimental values were within one standard deviation of this curve.

(4) The distribution of the sites from which the neutrons scatter was measured by removing the collimator in sections, starting with those farthest from the source. The curve so obtained was similar to that under point 3 but shifted toward larger contributions from those segments farther from the source.

(5) A time of flight spectrum was obtained for the neutrons scattered 2 cm into the opposite penumbra from one side of the collimator. The percentage of neutrons in the 12–14·8 MeV range, after correcting for detector efficiency, was 86·5%.

We conclude that most of the scattering occurs in the region closest to the source and that it can be significantly reduced by proper choice of collimator shape.

REFERENCE

1. E. P. Cytacki and C. A. Kelsey, Contribution of patient scatter to 15 MeV neutron beam penumbra. *Europ. J. Cancer* **10,** 329 (1974).

Europ. J. Cancer Vol. 10, pp. 333-334. Pergamon Press 1974. Printed in Great Britain

Dosimetry for Neutron Radiotherapy*

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THE DOSIMETRY of neutrons is more difficult than that of X- and gamma radiation and in many instances, including radiotherapy, the task of the radiological physicist is made more demanding by the requirement that both kinds of dosimetry must be carried out simultaneously. Nevertheless, the techniques required seem to be well in hand. Once the results of the ICRU intercomparison of neutron dosimetry are available, we should have a fair idea of the accuracy obtainable [1]. It may be expected that even in the unlikely case that substantial discrepancies are revealed, international agreement on neutron dosimetry standards should still be reached in a rather short period of time.

The subject of neutron dosimetry has been studied for a long time. Our laboratory developed the tissue-equivalent ionization chamber a quarter of a century ago. What is new is the employment of neutrons in the treatment of malignant disease. It is important that the radiotherapist be aware of the fact that in the case of neutrons, dosimetric information has implications that are somewhat different from those for electromagnetic radiations. Therapists have come to expect that effects, such as cell inactivation, increase rapidly with dose, and they have become equally accustomed to the notion that a given absorbed dose has essentially the same biological effect regardless of radiation energy. In the case of neutrons the reverse tends to be true in both instances. On one hand, biological effect is more likely to be proportional to dose, while it is much more dependent on radiation energy.

A number of investigators, and notably Barendsen, have shown that the biological effect of radiations depends strongly on LET. I believe we have now established that the basic dependence is one on the square of the LET. However, this is modified at both low and high LET values. At the low end, doses that are of importance in radiotherapy are sufficiently large, so that several particles are involved in the process of inactivation with the result that the differences in LET become unimportant. At the high end of the LET scale, saturation limits the effectiveness of even single particles and the RBE drops at LET values in excess of about 100 keV/μm. This already complex relation becomes even more involved for neutrons which generate secondaries having a wide range of LET's. The combined effect of these variables results in changes of RBE with neutron energy that are more moderate than those that can be obtained in track segment experiments. Nevertheless, the variation is certainly not negligible. Dr. Hall will present some data which indicate a more than twofold variation of RBE with neutron energy [2]. Even for a given neutron source, changes of the LET spectrum within the irradiated tissues may result in RBE variations that are not negigible. Utilizing information on the LET spectrum produced by 14 MeV neutrons near the surface, and at a depth of 10 cm in a human phantom, we have been able to calculate that the RBE varies by about 10%.

The energy dependence of RBE will make comparisons between therapy centres pre-

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carious even when they employ nominally equal neutron sources in different collimation arrangements. Results should probably not be correlated without prior detailed evaluations of LET spectra. Initially the primary function of dosimetry will be to assure consistency at any given institution. In this situation precision is more important than accuracy.

The RBE also depends on the type of tissue irradiated. Currently available information on this point is incomplete, but it would appear that an even more important variation is a steady increase of RBE with decreasing dose. This does not really reflect an increase in neutron efficiency, but is merely due to increasing inefficiency of electromagnetic radiation. However, it must be remembered that therapists are likely to rely on their experience with low LET radiation, which causes them to anticipate certain reactions in the normal tissues receiving a dose that is less than the tumour dose. They must be prepared for more severe reactions in the case of neutrons.

Another important related subject is that of radiation leakage. Difficulties in obtaining adequately strong neutron sources and the poor attenuation characteristics of even the best available materials make it difficult to interpose adequate shielding if patients are to be irradiated at an acceptable dose rate. There has been a tendency to permit radiation leakage outside the treatment field that is higher than that considered acceptable in X- and gamma ray therapy. The relatively high effectiveness of low neutron doses mandates the opposite course.

The OER is an even more sensitive function of LET. Nevertheless it is less dependent on neutron energy than is RBE. Here a complex compensatory set of mechanisms is responsible. Since OER has been invoked as one of the major reasons for neutron therapy, it is fortunate that it may be expected to differ comparatively little among the various neutron sources which are currently used or under consideration for therapy.

REFERENCES

1. L. J. Goodman, Problems in neutron dosimetry, *Europ. J. Cancer* **10,** 309 (1974).
2. E. J. Hall, RBE and OER values as a function of neutron energy. *Europ. J. Cancer* **10,** 297 (1974).

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Depth Dose Characteristics and Beam Profile Properties of Cyclotron-Produced Neutron Beams

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Abstract—*Fast neutron beams produced with cyclotrons are being used increasingly for radiation therapy. The depth dose characteristics, gamma contamination and beam profile properties of these beams depend on the energy and type of the accelerated particles as well as on the reaction utilized to produce neutrons.*

INTRODUCTION

THERAPEUTIC fast neutron beams should, ideally, have a penetration into tissue comparable to that of the X- or gamma radiation which would normally be used. If a less penetrating neutron beam is used, the biological advantage may be lost because a smaller effective dose would be given to a deep seated tumour. However, since neutron beams are invariably accompanied by gamma radiation, the proportion of which varies with, for example, depth in tissue and field size, it is desirable that both radiations should be estimated separately, a requirement which presents experimental difficulties.

For measurement of the two components outside the primary beam similar, but more severe, difficulties are encountered since the neutron energy spectrum differs from that in the primary beam. This paper discusses the depth dose and beam profile properties of some cyclotron-produced fast neutron beams. No attempt is made to discuss the limitations of the experimental techniques from which this data was derived.

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NEUTRON PRODUCTION WITH CYCLOTRONS

Fast neutron beams are usually produced with cyclotrons by bombarding a beryllium target with energetic deuterons, but other reactions are possible. The advantages of this reaction are that beryllium possesses good mechanical, chemical and thermal properties (but it does present handling problems due to its toxicity) whilst deuteron bombardment produces high neutron fluxes, the neutrons being emitted primarily in a narrow cone in the forward direction. This latter property assists in the achievement of high dose rates and simplifies collimation and shielding. However, since the width of the cone of neutrons decreases with increasing deuteron energy it might present problems of beam distribution with high energy cyclotrons when large field sizes are used.

A broad spectrum of neutron energies is produced which has a maximum intensity at, and a mean energy of, about 0·4 times the deuteron energy [1]. The dose rate in the forward direction varies approximately as E_d^b where E_d is the deuteron energy and $b = 2{\cdot}7$–$3{\cdot}0$ [2, 3].

DEPTH DOSES OF CYCLOTRON-PRODUCED FAST NEUTRON BEAMS

The depth doses of cyclotron-produced fast neutron beams depend upon such parameters as the energy and nature of the accelerated ion, the target material and its thickness, source-to-skin distance (SSD) etc. The most frequently used configuration is the bombardment with deuterons of a beryllium target which is thicker than the range of the incident ions. One exception to this is at the MRC Cyclotron where the target is thinner than the deuteron range but not thin enough to produce a significant increase in the neutron depth doses.

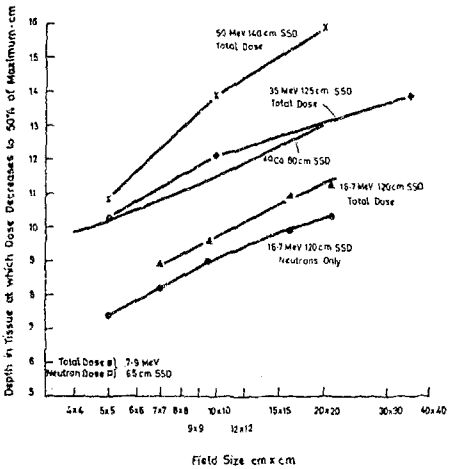


Fig. 1. Depth dose data of fast neutron beams produced by bombarding thick beryllium targets with deuterons. 50 MeV deuterons, Texas A & M Variable Energy Cyclotron College Station, Texas; 35 MeV deuterons, Naval Research Laboratory Cyclotron, Washington, D.C.; 16·7 MeV deuterons, Medical Research Council Cyclotron, London; 7·9 MeV deuterons, Memorial Sloane Kettering Cancer Center, New York.

In Fig. 1 the depth doses obtained by bombarding thick beryllium targets with deuterons (E_d = 7·9 MeV [4], 16·7 MeV [5], 35 MeV [6], and 50 MeV [7] are compared with those for cobalt-60 gamma rays under the conditions at which they are, or might be, used for radiotherapy. In selecting a suitable SSD for radiotherapy a compromise is necessary at low energies between the requirements for a suitable depth dose and adequate dose rate. Thus, at the higher energies where adequate dose rates are easily achieved improvements in depth dose result not only from the availability of higher energy neutrons but also from increased SSD. For the beams produced by 50 MeV and 35 MeV deuterons the curves represent total dose due to neutrons and gamma radiation. At 7·9 MeV [4] and 16·7 MeV an attempt has been made to separate the two components and curves are presented for total dose and neutrons only. At these energies the use of total dose overestimates the depth at which the neutron dose decreases to 50% by 5–10 mm. Furthermore, using thick beryllium targets bombarded with deuterons it appears that an ion energy of at least 30 MeV–35 MeV is required to obtain depth doses comparable to or in excess of those obtained with cobalt-60 gamma radiation.

Measurements of the build up of dose in the first few millimetres of tissue with neutrons produced by 16·7 MeV, 35 MeV and 50 MeV deuterons show that the maximum dose occurs at a depth of about 0·3 $R_p(E_N \text{ max})$ where $R_p(E_N \text{ max})$ is the range of protons whose energy is equal to the maximum energy of the neutrons produced (i.e. about 4 MeV greater than the deuteron energy). In tissue the maximum dose occurs at about 1·4 mm for neutrons produced by 16 MeV deuterons, 4·4 mm produced by 35 MeV deuterons and 7 mm produced by 50 MeV deuterons. For neutrons produced by 16 MeV deuterons it has been shown that this skin-sparing is significant clinically [8]. However in the build-up region the high LET components of the dose (i.e. α particles and heavy recoil nuclei) attain equilibrium in much smaller distances than the proton component and they contribute a higher proportion of the neutron dose as the neutron energy increase, while their RBE may be large. Thus dose measurements may underestimate the biological effect.

Estimates of the gamma contamination as a function of depth in tissue have been made with beams produced by bombarding a thick beryllium target with 7·9 MeV deuterons [4] and for the MRC Cyclotron (16·7 MeV deuterons). With the MRC Cyclotron the gamma dose for a 9·5 cm × 9·5 cm field is about 3% with no phantom present. However, when the beam interacts with tissue additional gamma radiation is produced and the ratio of gamma dose to neutron at the surface of a phantom increases to 6% for a 9·5 cm × 9·5 cm field, and to 8·5% for a 20 cm × 20 cm field. The ratio of gamma dose to neutron dose increases approximately linearly with depth in tissue to 24% for a 9·5 cm × 9·5 cm field and 36% for a 20 cm × 20 cm field at 20 cm deep. Using 7·9 MeV deuterons with a 5 cm diameter field at 65 cm SSD the corresponding value on

the surface of the phantom was 5% increasing approximately as the square of the depth to 36% at 20 cm deep.

Since large cyclotrons are required to produce depth doses comparable to those of cobalt-60 gamma radiation, it would be economically advantageous if more penetrating beams could be obtained from the smaller machines. By bombarding a thick beryllium target with 23·2 MeV ^{3}He ions at 65 cm SSD the total dose was found to decrease to 50% at 6·7 cm for a 5 cm dia field in comparison with 6·0 cm when 7·9 MeV deuterons were used from the same machine [4]. When neutrons only were estimated the corresponding figures were 6·2 cm and 5·7 cm respectively. The dose rate for the ^{3}He reaction was a factor of 2–3 less than that for the deuteron induced reaction and the ^{3}He induced reaction showed considerably more γ radiation (15% at the surface, 40% at 20 cm deep).

The use of beryllium targets thinner than the range of the incident deuterons has also been examined [9, 10]. The maximum penetration was found to occur when the thickness of beryllium was about $\frac{1}{4}$ of the range of the incident deuterons. With such a beryllium target bombarded with 16 MeV deuterons the depth at which the neutron dose decreased to 50% was 9·1 cm compared to 8·4 cm with a thick target for a 10 cm × 10 cm field at 100 cm SSD. However this increase in penetration was accompanied by a decrease of dose rate by a factor of about 2·2.

The use of target materials other than beryllium has also been examined [9, 11]. Lithium and carbon both produced neutron depth doses similar to those obtained with beryllium but beryllium produced the highest dose rate. A heavy water target bombarded with 16 MeV deuterons produced depth doses comparable to those obtained with the optimum thin beryllium target whilst with 11 MeV deuterons incident upon heavy water the depth dose was comparable to that obtained by bombarding a thick beryllium target with 16 MeV deuterons. However within the range of energies studies (10 MeV–16 MeV) the neutron dose rates with heavy water were only about half those obtained with a thick beryllium target.

RADIATION OUTSIDE THE PRIMARY BEAM

The neutron energy spectrum outside the primary beam differs from that within the beam due to scattering in the shielding materials and the patient. Figure 2 shows measurements using activation detectors made in air with the MRC Cyclotron along an axis perpendicular to the beam at 120 cm from the target. The distances shown for shielding materials are projections of the actual distances onto the line of measurement. Where the beam primarily penetrates the wooden collimators the data suggests that the energy spectrum is less peaked than the primary beam since the detectors with the highest and lowest activation thresholds show higher counting rates relative to the central axis than those with thresholds of a few MeV. However, in the region where most of the attenuation is due to steel the reactions with the highest threshold energies produce steeper curves consistent with the inelastic scattering properties of steel, and at 50 cm from the central axis it appears that there is a considerable increase in low energy neutrons compared with the primary beam.

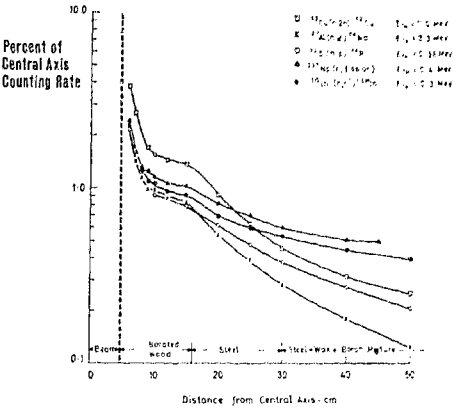


Fig. 2. Neutron beam profile measurements with various threshold detectors in air. Field size 9·5 cm × 9·5 cm. Medical Research Council Cyclotron

Such changes in the neutron spectrum enhance the problems of separating the radiation outside the primary beam into its two components. In air, total doses of 2–4% of the dose in the primary beam can be achieved a few centimetres from the edge of collimators 50–75 cm long [4, 5, 6]. With a phantom in position a similar contribution results from scatter. However, measurements with the beam from the MRC cyclotron indicate that on the surface of the phantom at about 10 cm from the central axis of a 7 cm × 7 cm field 50% of the total dose is due to gamma radiation whilst at 70 cm from the axis the total dose is 0·9% of which 75% is due to gamma radiation. At 10 cm deep in the phantom at the same dis-

ances gamma radiation contributes 60% and 40% of the total dose respectively.

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REFERENCES

1. C. J. Parnell, A fast neutron spectrometer and to use in determining the energy spectra of some cyclotron-produced fast neutron beams. *Brit. J. Radiol.* **45,** 452 (1972).
2. C. J. Parnell, G. D. Oliver, P. R. Almond and J. B. Smathers, The dose rate of cyclotron-produced fast neutrons beams. *Phys. in med. Biol.* **17,** 429 (1972).
3. L. S. August, R. B. Theus, F. H. Attix, R. O. Bondelid, P. Shapiro, R. E. Surratt and C. C. Rogers, Fast neutron dose rate vs incident deuteron energy for $d + {}^{9}Be$. *Phys. in med. Biol.* **18,** 641 (1973).
4. J. S. Laughlin, T. R. Canada, L. D. Simpson, J. C. McDonald, T. Y. T. Kuo and A. Mittleman, Compact cyclotron neutron dosimetry. Presented at the *German–Austrian Roentgen Congress*, Vienna, April, 1973.
5. D. K. Bewley and C. J. Parnell, The fast neutron beam from the MRC Cyclotron. *Brit. J. Radiol.* **42,** 281 (1969).
6. C. C. Rogers and F. H. Attix. (Private communication).
7. P. R. Almond. (Private communication).
8. M. Catterall, C. Rogers, R. H. Thomlinson and S. B. Field, An investigation into the clinical effects of fast neutrons. *Brit. J. Radiol.* **44,** 603 (1971).
9. C. J. Parnell, An examination of some methods of improving the depth dose characteristics of cyclotron-produced fast neutron beams. *Brit. J. Radiol.* **44,** 612 (1971).
10. L. J. Goodman, S. A. Marino and J. T. Brennan, Minimum cyclotron size for radiation therapy; a study of relevant physical parameters. In *Uses of Cyclotrons in Chemistry, Metallurgy and Biology* pp. 209, Butterworths, London (1970).
11. C. J. Parnell, B. C. Page and M. A. Chaudhri, A comparison of heavy water and beryllium as cyclotron targets for fast neutron production for radiotherapeutic applications. *Brit. J. Radiol.* **44,** 63 (1971).

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Developments in Achieving Optimum Beam Profiles for 15 MeV Neutron Beams

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Abstract—*The basic problems and principles involved in designing bulk shielding and collimation for a 15 MeV neutron generator are discussed. Even with shields up to 60 cm thick, the average dose level, outside the beam in a scattering medium is of the order of 1% of the dose in the centre, this result being a consequence of basic difficulties in designing neutron collimators, and of scattering of radiation from the main beam itself. The quality of this stray radiation is discussed and it is shown that it is predominantly γ-radiation.*

INTRODUCTION

THE PROBLEM of confining the output from a deuterium–tritium neutron generator to a beam which is sufficiently well defined for radiotherapy is a formidable one for the following basic reasons:

1. The differences in the attenuation properties of the different elements for fast neutrons are relatively small. The large difference between the linear attenuation coefficients for lead and tissue for X-rays makes it relatively easy to design shields for X-rays which readily penetrate a patient, but the differences for fast neutrons are much less useful in this sense.

2. The materials in the neutron shield are likely to be activated and this raises radiation protection problems for staff who have to work near the generator when the neutron output is turned off.

3. For a D–T neutron generator the neutrons are emitted isotropically from the target. While this has the advantage that it is possible to have large field sizes, it has the disadvantage that equal thickness of shielding is required in all directions.

4. The thickness of shielding that can be used is limited by the relatively low outputs that are available from this type of generator. There is also a desire to minimize the size and weight of the shielding so that the system can be made mobile.

5. The neutron sources or targets are several cm dia and this not only gives a relatively large penumbra with any particular collimator, but increases the problem of oblique penetration of the collimator walls.

The design of the shield for a neutron source falls naturally into two halves, the main or bulk shield, and the collimator and these will be considered separately.

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BULK SHIELDING

The attenuation of broad beams of 15 MeV neutrons has been studied experimentally by Broerse and van Werven [1], Greene and Thomas [2], Greene and Major [3] and Maruyama and Bouts [4]. Computer calculations have been published by Grünauer [5] and Hehn and Becker [6]. Even if the radiation incident on the attenuating material consists of primary neutrons only, that measured on the exit side will consist of primary and scattered neutrons and γ-rays. The experimental work mentioned has taken account of

this, but the computer studies give information about only the neutron component of the transmitted dose.

It is generally agreed by the authors quoted that iron is the best material for attenuation of the primary neutrons, but it is also necessary to include some hydrogenous material to deal with scattered neutrons from the iron, and an outer layer of high atomic number material to attenuate γ-rays generated in the shield itself. Three layer systems, and multilayer systems have been investigated, with the result that for a 50 cm thick shield, the total transmitted radiation (primary and scattered neutrons and γ-rays) will be between 1% and 2% of the primary level. While tungsten might have some advantages over iron for primary neutron shielding [6, 7], considerations of cost, weight and difficulties of fabrication rule this material out for bulk shielding.

The main activity produced in the shielding is from ^{56}Mn, a γ-ray emitter with a half life of 2·6 hr. Lundberg [8] has discussed the effect of impurities in mild steel and has suggested that problems arising in this way may be avoided by using Swedish iron. Since radioactivity produced in the shield is concentrated on the target side, the main structure is largely self shielding for the γ-ray production, except near the collimator hole.

COLLIMATOR DESIGN

Because of the large thickness of attenuating material required to attenuate neutrons moving in unwanted directions, it is immediately clear that interleaved collimators to give continuously variable field sizes are impracticable.

The authors previously quoted [3, 5–7] have produced experimental and theoretical studies on the design of interchangeable fixed field collimators, and these may be discussed under the following headings: [1] choice of materials; [2] collimator geometry and [3] measurements on collimated beams.

1. *Choice of materials*

The materials which give the best beam definition are essentially the same as those which give optimum bulk shielding and the same considerations which lead to the use of successive layers of iron, polythene and lead apply. The activation problems already discussed are however more acute for collimator design because (*a*) the activity generated at the target end of the system results in a γ-ray beam emerging from the collimator when the neutron output is switched off; (*b*) when collimators are being changed, or in store, operators may be directly exposed to the γ-rays from the target end of the collimator. The latter problem can be solved by the use of a remote handling system which removes the collimator from the machine and places it in a shielded room. This method is used for the Radiation Dynamics installation at Hamburg. Alternatively both problems may be dealt with by the use of materials which minimize the activation, and Knight *et al.* [7] have used tungsten instead of iron in collimators for this reason. As previously stated, tungsten may be a better material than iron as a primary beam attenuator and in the present context has the additional advantage that it produces much less radioactivity.

2. *Collimator geometry*

Collimator lengths are, as with the thickness of bulk shielding, limited by available neutron outputs and 40–60 cm seems to be accepted as the practical range. Most collimators discussed in the literature have walls which converge linearly onto the neutron target, and there is no evidence that more complex shapes (e.g. the use of wide throats at the target end) have any advantage. From the point of view of optimum beam definition it is clear that the primary neutron attenuation should extend as close as possible to the target, but this will also maximize any radioactivity produced. The use of linings (i.e. the construction of collimators whose inside walls are made entirely of steel or tungsten) has been considered as a method of limiting oblique penetration by primary neutrons but any advatange is probably marginal.

3. *Measurements on collimated beams*

The radiation levels outside a 15 MeV neutron beam must be of the order of 1% of that at the beam centre. This is high compared to that achieved with X-ray collimators. For this reason it is important not only to make the usual dosage measurements inside the beam and at its edges, but also to examine the dose levels for distances comparable to the length of a patient's trunk.

A result of this type of measurement is shown in Fig. 1, where the beam profile measurement for a 10×10 cm field in air has been extended to 50 cm from the beam centre. These measurements were made on the beam from the Elliott "Hiletron" 15 MeV neutron generator, where the 60 cm long collimator is made from the tungsten–polyethylene–boron mixture described by Knight *et al.* [7]. The results are expressed as percentages of the

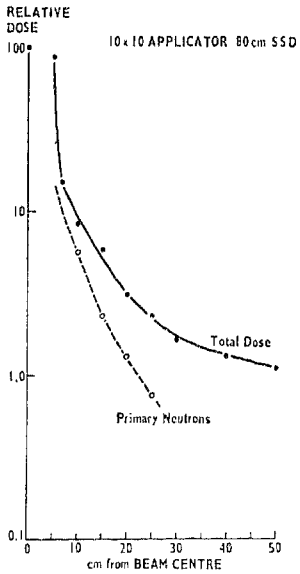


Fig. 1. Beam profile given by the "Hiletron" neutron generator, in air for a 10 × 10 cm neutron field at 80 cm from the target

total dose at the beam centre as measured with a polyethylene–ethylene ionization chamber, and some measurements of the relative primary neutron dose, as measured with a suitably biased knock-on proton detector are also shown. It can be seen that (*a*) the dose level inside the beam is reasonably uniform, (*b*) the dose falls away adequately sharply to the 10% level and (*c*) outside this level, while the primary neutron dose goes down rapidly, the total radiation dose is still relatively high.

The beam profile will change both in quantity and radiation quality in a scattering medium and Fig. 2 shows some data for the same field size measured at a depth of 10 cm in a phantom. Here the total $(n+\gamma)$ dose, and the γ-ray dose are expressed as percentages of the total dose on the beam centre and it can be seen that the proportion of the dose due to γ-rays increases rapidly outside the beam edges. For comparision the dose levels outside a 10 × 10 cm 250 kV X-ray beam, at the same depth in a phantom, are shown.

There remains the question of radiation quality and its variation inside the main beam. The literature shows that the γ-ray content of the beam increases slightly with depth in the phantom. The detailed way in which this happens probably depends on the particular type of collimator used. The collimator used for the measurements quoted gives a γ-ray content of the beam in air of 12%. We think this increases fairly rapidly over the first few cm to about 15% and then increases rather slowly thereafter. The detailed way in which these variations, and variations in the neutron spectrum occur will depend on the "in air" spectrum of the collimated beam. A "pure"

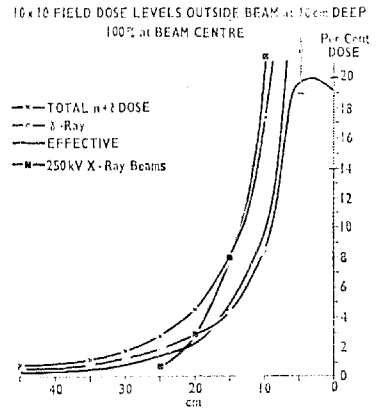


Fig. 1. Beam profile for a 10 × 10 cm field, at 10 cm depth in a phantom. The results are given as total dose, γ-ray dose and effective dose, defined as neutron dose + ⅓ γ-ray dose. A comparable profile for a 10 × 10 cm 250 kV beam, at 10 cm depth in a phantom is also shown

beam, i.e. one with primary neutrons only, will in fact show more spectral variations than a contaminated beam, containing γ-rays and scattered neutrons originating outside the phantom.

CONCLUSION

A 15 MeV neutron beam in a scattering medium cannot be as well defined as that from an X-ray generator and therefore the average body dose to a patient receiving a neutron exposure will be higher for this type of treatment. This increase in total-body dose is due partly to the difficulties of collimator design discussed here, and also to the effect of radiation scattered from the main beam [9].

REFERENCES

1. J. J. Broerse and F. J. van Werven, Shielding measurements for 3 MeV and 15 MeV neutrons. *Hlth. Phys.* **12,** 83 (1966).
2. D. Greene and R. L. Thomas, The attenuation of 14 MeV neutrons in steel and polythene. *Phys. in med. Biol.* **14,** 45 (1969).
3. D. Greene and D. Major, Collimation of 14 MeV neutron beams. *Europ. J. Cancer* **7,** 121 (1971).
4. T. Maruyama and C. J. Bouts, Attenuation of 15 MeV neutrons in multilayer shields composed of steel, polyethylene and borated materials. *Phys. in med. Biol.* **17,** 420 (1972).
5. F. Grünauer, Calculation of a neutron collimator for therapeutical purpose. In *Proc. 1st Symp. Neutron Dosimetry in Biology and Medicine*, Munich, (Edited by G. Burger, H. Schraube and H. G. Ebert), p. 511 (1972). Commission of the European Communities, Luxembourg.
6. G. Hehn and R. Becker, Optimization of a collimator for 14 MeV neutrons. In *Proc. 1st Symp. Neutron Dosimetry in Biology and Medicine*, Munich. (Edited by G. Burger, H. Schraube and H. G. Ebert), p. 529 (1972). Commission of the European Communities, Luxembourg.
7. A. Knight, D. A. Lundberg and T. O. Marshall, The development of a 15 MeV neutron beam collimator for radiotherapy. In *Proc. 1st Symp. Neutron Dosimetry in Biology and Medicine*, Munich. (Edited by G. Burger, H. Schraube and H. G. Ebert), p. 541 (1972). Commission of the European Communities, Luxembourg.
8. D. A. Lundberg, The design of a practical fast neutron therapy equipment for routine clinical use. *Brit. J. Radiol.* **44,** 708 (1971).
9. 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,** 129 (1971).

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The Treatment of advanced Cancer by Fast Neutrons from the Medical Research Council's Cyclotron at Hammersmith Hospital, London

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Abstract—*Patients continue to be treated regularly three times weekly on the Medical Research Council's cyclotron. A standard dose of* 1440 *rad in* 12 *treatments over* 26 *days is given. All the patients have had such advanced or "radioresistant" tumours that it was thought that other methods of treatment would not be satisfactory, or the tumours had recurred. Two hundred and thirty-eight patients completed their treatment by January* 1973 *and* 58 *lived with no sign of tumour in the neutron treated area for more than one year. One hundred and thirty-five who survived for less than one year, died of metastases but with regressing or completely regressed tumours in the neutron treated area.*

Forty-five tumours probably recurred and were all associated with doses which were lower than the standard dose. Necrosis of the skin appeared in 11 *of* 97 *patients surviving more than* 6 *months, but in each of these cases, there was a precipitating factor and the necrosis was never unexpected or unexplained.*

Noteworthy results have been achieved in tumours of the salivary glands, buccal cavity, oropharynx, nasopharynx, stomach and in sarcoma and fixed glands invaded with adenocarcinoma, melanoma and squamous cell carcinoma.

The effects of neutron therapy on the normal tissues which are unavoidably irradiated are being carefully observed. The eye is a uniquely good site for recording basic radiation changes and valuable information is being collected.

INTRODUCTION

SINCE NOVEMBER 1969, the fast neutron beam from the Medical Research Council's Cyclotron has been used regularly three times weekly for the treatment of selected patients with advanced but localised cancer. The beam is reliable and is only not available for 1 week every 6 months for maintenance requirements. The output is good and treatment times per field are of the order of 4 minutes. Several special techniques have been developed as reported elsewhere [1–3]. These include the activation of pure aluminium to check the dose received at various sites in the treated area, the taking of neutrograms during treatment for use as check films, the lining of masks and bolus with a thin sheet of lead to restore skin sparing and a plastic for bolus. Wedged filters of 24° and 45° in polystyrene are used. Although the collimated fields are all squares or rectangles, accurate protection of various parts of the field is possible by a wide variety of borated wooden shields which can be inserted into the field.

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The greatest technical disadvantage is the energy of the beam which delivers 50% of the dose at 8 cm depth in tissue compared with 70% at the same depth from the 6 MeV linear accelerator. This relatively poor penetration results in inhomogeneity of dose throughout large tumours and in high doses being received by some normal tissues when wedged fields are used, especially when fields are greater than 10×10 cm. It also requires the use of 4 beams for internal tumours and makes it impossible to treat these except in very thin patients. The fixed horizontal beam is also a technical disadvantage but can be partly overcome by flexibility of the positioning of the patient in the vertical or horizontal plane.

The standard dose is 1440 rad given in 12 treatments over 26 days. If the overall time has to be prolonged or the number of fractions altered, the total dose is modified according to the formula $T.D. = NSD \times N^{\cdot 04} \times T^{\cdot 11}$ [4]. A factor for reducing the dose to large areas is no longer used.

THE PATIENTS

Since November 1969, 360 patients have been considered for fast neutron therapy. They were all thought, by the referring consultants to be unlikely to respond satisfactorily to X- or gamma rays because of the size, local extension or histology of the tumours or because the tumours had already recurred after surgery, chemotherapy, hormone therapy or radiotherapy or a combination of these methods. They have therefore been a selected group, with extremely poor prognosis. Forty-five were too ill for anything except terminal care or were technically unsuitable for treatment and in 27 others, the full course of treatment could not be completed because of deterioration of the general condition. No patient under the age of 17 years has been treated.

Two hundred and thirty-eight patients completed their treatment more than 6 months ago and this paper reports their survival, tumour regression and recurrence and the complications of treatment.

RESULTS

General review

One hundred and forty-one patients died less than 6 months after treatment and 39 others died 6–12 months afterwards. In most, the cause of death was generalised metastases, but some died of cardiovascular and other diseases. In 135 of these, the neutron treated umours were either regressing at the time of death or had completely regressed.

There was complete regression of tumour for 1–2 years after treatment in 34 patients, of whom 12 had adenocarcinomas. These were in the breast in 4, the large bowel in 4, the stomach in 2, pancreas in 1 and nasopharynx in 1. Eight others had stage T3 or T4 well differentiated squamous cell carcinomas of the buccal cavity. Six patients had sarcoma, 2 had melanoma, 2 had salivary gland tumours, 2 had astrocytomas, grade 3 or 4, and 2 had transitional cell carcinomas.

The tumours of 18 other patients showed complete regression for 2–3 years and these included 6 with adenocarcinoma, which was in the breast in 3, the large bowel in 2 and stomach in 1. Three patients had well differentiated squamous cell carcinomas of neck nodes, cervical oesophagus and buccal cavity. Three others had sarcoma, another had melanoma, 4 had salivary gland tumours and 1 had an astrocytoma grade 3.

Six patients lived with no tumour in the treated area for more than 3 years and consisted of 2 with adenocarcinoma of the breast, 1 with a squamous cell carcinoma of the lung, 2 with T3 squamous cell carcinomas of the skin and 1 with a mucoepidermoid carcinoma of the parotid gland. Thirty-five of these 58 patients are alive.

Twenty-two tumours have definitely recurred or were residual when the patient died. Eleven of these were in the cervical nodes and were technically extremely difficult to treat and therefore did not receive the full dose. These patients had kyphosis or had undergone cervical node dissections with subsequent recurrence and fixity or distortion of the neck. Five others had intra-abdominal tumours, the extent of which was, initially, uncertain and the situation of which made it impossible to treat with a homogeneous dose. One patient was treated when the field size factor was being applied and had the dose reduced by 8% from the standard. Four others had their treatment over a protracted time with the dose modified according to the formula already stated.

Twenty-three other patients possibly had recurrent or residual tumours. The uncertainty was due to the site in 22 where the tumour could not be properly observed, 17 of these patients had intra-abdominal tumours and in 5 others it was in the brain. One patient was examined in her own home and the assumed recurrence may have been fibrosis.

Necrosis of the skin and subcutaneous tissues has appeared in 11 of the 97 patients surviving more than 6 months after treatment. Seven of these suffered from very large sarcoma, with skin infiltration and ulceration in 4, and 3 patients had already received a full course of low LET radiation in the same region. Four other patients had adenocarcinomas in the breast, stomach or rectum which were the sites of previous surgery or trauma or infection, and also required large fields of irradiation to cover the tumours. No area of necrosis appeared without a precipitating factor.

The detailed results of patients with tumours of the buccal cavity, the breast, oesophagus and stomach and of melanoma will be the subject of papers in specialist journals. In this short general report, the results of treatment of only two groups will be given.

Salivary gland tumours

Twelve patients suffered from these tumours, of which 5 were adenoid cystic, 3 mucoepidermoid, 1 adenocarcinoma and 2 were poorly differentiated adenocarcinoma. One patient with an adenocarcinoma had received 5000 rad of gamma rays from cobalt-60 but the tumour recurred and was fungating when neutron therapy was given. All the tumours were fixed deeply, and 7 were also invading the skin. Eight of the tumours could be contained within two fields of 100 cm^2 but in four, the tumours were very large and required fields of 170 cm^2. Pain was a dominant symptom in 11 patients.

Pain, trismus and fungation when present were relieved in all cases. There had been complete paralysis of the 7th nerve in three patients for more than a year and this was improved in only one. In one patient, where this had been present for only 2 months, neutron therapy restored this function when the tumour regressed.

There was complete regression of the tumour in every case.

Depilation of the treated skin occurred in every case but there was no necrosis of skin, bone or subcutaneous tissue. Dry mouth and diminution of taste lasted for about 3 months after treatment and returned nearly to normal in each case.

None of the tumours has recurred and the follow up periods since treatment have been 3 years (1 case) $2\frac{1}{2}$ years (2 cases) and $1\frac{1}{2}$ years (2 cases). Two patients died of metastases with no sign of tumour in the treated area, one year after treatment and 2 others after six months. Two patients were treated only 3 months ago and at present are well with no sign of tumour.

The patient whose adenocarcinoma recurred after cobalt treatment died of metastases 5 months after neutron therapy. Autopsy revealed no tumour in the treated area to which a skin graft had been successfully applied.

Neutron therapy benefited all the patients treated and there were no severe side effects.

Sarcomas

Twenty patients with very extensive sarcomas were treated, all of which involved adjacent skin or bone. Five of these tumours were well differentiated fibrosarcomata, 2 were leiomyosarcoma, 2 chondrosarcoma and 2 osteosarcoma. Three of these patients had tumours which had recurred after a previous full course of supervoltage radiation.

Six others had rather poorly differentiated fibro or liposarcoma, which were extremely large and advanced.

When neutron treatment was started, these patients hd no sign of metastases and pain, fungation and large masses were the main symptoms. In five, the sacral area was involved, in 2 the antra and in the rest the tumours were in the groin, thigh or leg.

None of these 20 tumours failed to regress, but this was incomplete in 4 patients who died of metastases less than 6 months after treatment and whose tumours were in fact reducing in size at this time and biopsies in one, showed no tumour. Complete regression, clinically, occurred in 16 and this was confirmed by biopsy in 5. One patient's leg was amputated and a few recognisable tumour cells were seen in the bone but there were none in the soft tissues. At autopsy in another case, some residual cells were seen in the bone, but not in the soft tissues. Six patients are alive and well 10–30 months after treatment. Pain was relieved completely in every case.

Complications were most frequent in this group and consisted of necrosis of skin and subcutaneous tissues. This occurred in 6 patients whose tumours were infiltrating or frankly ulcerating the skin before treatment and were very large. Two or three wedged fields of 21×21 cm each were required for most of the tumours and resulted in strips of tissue receiving higher doses than the standard dose. Previous surgery and radiotherapy, or both, had devitalised the tissues in 3 patients. A skin graft was successfully applied to one of these and he remains well and tumour free 22 months later.

MEASUREMENT OF THE EFFECTS ON NORMAL TISSUES

In conjunction with specialists in various clinical disciplines such as gastroenterology and ophthalmology, accurate observations and measurements are being undertaken to compare the effects of different doses of high and low LET radiations. The eye is a uniquely suitable site for the observation and recording of basic radiation changes since its many different structures are exposed to observation with the appropriate instruments. Techniques have been developed by Dr. Nicholas Brown of the Institute of Ophthalmology for biomicroscopic observation and quantitative recording of the ocular signs [5, 6]. Microphotography of the anterior segment of the eye and quantitative slit image photography of the lens are yielding valuable information on radiation induced changes; these are related to the precise dose received by various segments of the eye and ascertained by aluminium activation.

DISCUSSION

At the start of the present investigation, three and a half years ago, with the fixed horizontal beam and 7 MeV neutrons, a twofold policy was adopted. Firstly, a wide range of tumours was treated which were thought to be unlikely to respond to any other form of treatment and included many different histological types. Secondly a standard dose, fractionation and overall time has been given. The experience thus gained has resulted in a more precise definition of the type and site of tumours which can be treated beneficially and in an assessment of the range of doses which can be tolerated.

Considering the large sizes and "radio-resistance" of the tumours treated, it is perhaps surprising that more than a small percentage of patients received any significant regression of their tumours or relief of symptoms. However, of the 238 patients who completed their neutron therapy by January 1973, or six months ago, there was no relief of symptoms in only 15. Seven of these had inoperable carcinoma of the stomach and were complicated by metastases outside the treated area. Eight other patients had tumours which had recurred after a previous full course of low LET radiation and extensive surgery. Pain was relieved in practically every case and so was fungation. Fifty-eight patients lived for more than a year after treatment with complete regression of their tumours and only 5 of these recurred. In 45 others, the tumour was probably residual or recurrent at death, but these were all associated with uneven doses which were low at the site of the recurrence. None of the tumours which could be assessed was unchanged or enlarging two months after the end of treatment.

There is no consensus as to which, if any, tumours are resistant to low LET radiation and complete regression is occasionally seen in advanced stages of evey histological type. Randomised clinical trials have to be done to ascertain statistically the optimum treatment for a particular stage and type of tumour. Until the results of such trials are available, comparisons can only be made with previous clinical experience. Within the limitations of these comparisons, the regressions caused by fast neutrons to advanced tumours in the following sites have been remarkable—the salivary glands, buccal cavity, oropharynx, nasopharynx and stomach. Sarcoma in various sites and lymphatic glands invaded with adenocarcinoma, melanoma and squamous cell carcinoma have also responded well.

The numbers of salivary gland tumours and sarcoma which have been treated are fairly small, but the consistency of tumour regression and the completeness of relief of pain has been noteworthy.

All the salivary gland tumours were inoperable, but their volume was amongst the smallest of all the tumours treated. These tumours usually only respond partially to X-rays and the total regression in each of the 12 treated may be in part due to their relatively small size and their accessibility to the delivery of a uniform dose of neutrons. If this is so, better results might be expected if other tumours of relatively small size were treated or if a beam of neutrons with a higher energy was available so that deep seated tumours could be irradiated with a uniform dose.

The response of sarcoma to X- and gamma radiation is very variable and it is conceivable that all these patients would have had an equally effective clearance of their tumours with conventional forms of radiotherapy. Twelve died of generalised metastases less than 1 year after neutron therapy so that the follow up is short and the tumours may recur. However, the fact that all these tumours responded to neutrons, that none has recurred so far and that 2 regressed completely after previous radiation had failed, compares well with results obtained with chemotherapy, surgery and combined methods of treatment.

The very advanced stage of the tumours treated has caused many early deaths from

metastases and conclusions about late effects cannot yet be drawn. The results to date indicate however that unacceptable early side effects can be avoided, provided that scrupulous attention is paid to details of dose, that treatments are very carefully planned and are meticulously set up on each occasion.

The areas of necrosis which appeared were in no case unexpected or unexplained and were associated with one or more of the following precipitating factors—previous radiation or surgery, infiltration or ulceration of the skin with tumour, very large tumours requiring large fields which resulted in uneven distribution and "hot" spots of about 2000 rad, lack of care of the skin involving infection or trauma and the groin region in obese patients. The uncomplicated healing of the skin of laparotomy scars in 3 others indicated that satisfactory repair of skin can be expected in the absence of a precipitating factor and within the standard range of doses used.

The results from this application of cyclotron neutrons continue to be encouraging and are contributing to the growing interest in the world of fast neutron therapy. It is probable that with bigger cyclotrons with more penetrating beams and more flexible techniques, these results will be improved and the range of tumours accessible to treatment will be increased. It is important however to recognise the marked differences in clinical effects that exist between high and low LET radiation. The needs, which have already been stated [7], for training in neutron therapy as well as for reliable machines and good techniques must be emphasized. Without these, fast neutrons could be put at technical and clinical disadvantages which could give misleading information about their clinical value. The effects on the normal tissues which are unavoidably irradiated must also be studied and, for this, the collaboration of experts in various clinical disciplines is invaluable.

REFERENCES

1. D. K. Bewley, M. Catterall and B. C. Page, Medical radiography with fast neutrons. *Brit. J. Radiol.* **46,** 24 (1973).
2. M. Catterall, C. R. Rogers, H. R. Thomlinson and S. B. Field, An investigation in the clinical effects of fast neutrons, methods and early observations. *Brit. J. Radiol.* **44,** 603 (1971).
3. M. Catterall, The treatment of patients with fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital, London. In *Proc. Conf. Particle Accelerators in Radiation Therapy,* Los Alamos. United States Atomic Energy Commission, Oak Ridge, p. 135 (1972).
4. S. B. Field, An *in vivo* dosimeter for fast neutrons. *Brit. J. Radiol.* **44,** 891 (1971).
5. N. Brown, An advanced slit image camera. *Brit. J. Ophth.* **56,** 624 (1972).
6. N. Brown, Quantitative slit image photography of the lens. *Trans. Ophth. Soc. U.K.* **92,** 303 (1972).
7. M. Catterall, Clinical experience with fast neutrons. *Proc. roy. Soc. Med.* **65,** 839 (1972).

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RBE Values of 15 MeV Neutrons for Responses of Pulmonary Metastases in Patients

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Abstract—*Pulmonary metastases in 13 patients have been irradiated with single doses of 15 MeV neutrons or ^{60}Co gamma rays. Volume changes were measured from chest radiographs. In patients with multiple metastases, doses of neutrons and gamma rays were applied to different metastases. In patients with metastases in one lung only, the response to neutrons was evaluated first and gamma rays were given after the metastases had regrown to the pre-irradiation volume. From the measured volume changes for different types of tumours, the effectiveness of both radiations was evaluated and RBE values of 15 MeV neutrons were derived. RBE values ranged from 1·2 to 4·0 and showed a correlation with the growth rate of the lung metastases. These data indicate that 15 MeV neutrons could provide an advantage for slowly growing tumours.*

INTRODUCTION

IN A PAPER presented at the first meeting on fundamental and practical aspects of the application of fast neutrons in clinical radiotherapy, it was pointed out that for the evaluation of possible advantages of new treatment methods, which are expected to provide only a moderate gain in tumour cure rate, several problems have to be considered concerning the selection of types of tumours and the analyses of reponses [1]. A discussion of these problems with respect to fast neutron therapy led to the conclusion that before carefully controlled clinical trials are started, a significant effort should be made to obtain RBE values of fast neutrons for reactions of various types of tumours in patients. RBE values can only be derived using cases which provide possibilities for a direct quantitative analysis of responses. Pulmonary metastases of tumours have been shown to provide a clinical model which fulfils various requirements for such quantitative studies [2]. This model has now been employed for the determination of RBE values of 15 MeV neutrons compared to ^{60}Co gamma rays administered to 13 patients, of which some have been treated more than once.

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VOLUME CHANGES OF PULMONARY METASTASES AFTER IRRADIATION

In a fraction of the patients with tumours which have spread to the lungs, the metastases exhibit a spherical shape on radiographs and retain this shape during a considerable period of growth. This provides the possibility to measure their volume increase as a function of time and to derive the volume doubling time T_d. In a first series of metastases analyzed, the data obtained were generally consistent with exponential growth [2]. From measurements of pulmonary metastases from primary tumours for which the histological diagnosis is known, an insight can be obtained into the relation between the growth rate and the pathological nature of the tumours [3].

After irradiation of a metastasis with single or fractionated doses, the volume generally decreases, followed later by resumption of growth, whereby the extent of the volume decrease and the length of the growth delay is dependent on the dose given [4, 5]. For the data to be discussed in this paper, the volume doubling times of each metastasis was derived before irradiation from at least three, but usually many more radiographs of the chest in the posterior–anterior projection. After irradiation with a single dose of gamma rays or neutrons, the volume of the metastases decreased during 3–8 days, followed by an increase which frequently showed initially a more rapid growth rate, returning subsequently to the pre-irradiation value. By extrapolation of this latter part of the post-irradiation growth curve to the time of irradiation, an "effective volume reduction" or "extrapolated residual volume" (V_{res}) relative to the volume at the time of irradiation, can be derived [4, 5]. This volume reduction is not a direct expression of cell reproductive death, but is related in a complex way to the number of surviving cells and their proliferation characteristics as well as to cell death and removal of desintegrated cells. Nevertheless, the volume reduction can be used to derive a quantitative measure of the effect of irradiation. The parameter which is directly derived from the measurements, is the "growth delay" (T_{GD}), defined as the time in days required after irradiation to attain the pre-irradiation volume. The extrapolated residual volume (V_{res}) is related to the growth delay (T_{GD}) in days by the formula:

$$\ln V_{res} = -\ln 2 \cdot T_{GD}/T_d.$$

From former studies and also from the present data, the deduction was made that, with respect to responses to X-rays or gamma rays, it is possible as a first approximation to distinguish groups of tumours according to increasing volume doubling times. Thus tumours with volume doubling times (T_d) of 10–20 days, 20–40 days, 40–80 days, 80–160 days and more than 160 days have been taken as separate groups. For these groups curves have been derived which represent mean values of "extrapolated residual volume" as a function of the dose [5].

If two or more metastases are present, their growth rates and responses to the same dose of radiation are generally closely similar [4, 5]. Thus, a comparison of the effectiveness of 15 MeV neutrons with gamma rays is possible by irradiation of one metastasis with neutrons and the other with gamma rays. If only one metastasis is present, the derivation of an RBE is possible by first irradiating with a dose of one type of radiation, followed by a dose of the other type after this metastasis has returned to its pre-irradiation volume. If only a dose of 15 MeV neutrons could be given, an RBE value was derived by assuming that the response to gamma rays would have been similar to that of the group with corresponding volume doubling times.

In view of the stringent conditions that have to be fulfilled for patients with pulmonary metastases in order to consider them suitable for single-dose irradiation and because of ethical problems involved, over a period of 1·5 years only 13 cases could be obtained, which yielded reliable RBE values, although more patients were irradiated.

IRRADIATION TECHNIQUES AND DOSIMETRY

Fifteen MeV neutrons were produced by bombarding a rotating tritium target with 270 keV deuterons at a current of 6 mA from the electrostatic accelerator at Rijswijk. A fixed horizontal beam was employed with a target-to-skin distance of 45 cm. The patients were only suitable for fast neutron irradiation if they were in good physical conditions since the irradiations lasted up to 20 min and the patients were exposed in an upright position. For each patient the positions of the metastases in the lungs were determined from radiographs in two directions or from transversal tomograms.

On the basis of this information a depth dose curve was made, using data from phantom measurements [6]. Irradiations were carried out bilaterally. The neutron doses given in Table 1 include the gamma ray contribution which comprizes at most 10% of the total dose. The total dose is estimated to be accurate to within 10% and to be reproducible to within 5%. Doses of ^{60}Co gamma rays were used as low-LET radiation in the present series. These irradiations were carried out at the Netherlands Cancer Institute and at the University Hospital in Utrecht. A source-to-skin distance of 50–60 cm was used to obtain depth–dose characteristics similar to those of the neutron irradiations: No correction for air in the lungs was made. In former studies and in experimental investigations 250 kVp X-rays were generally used. In order to derive the RBE values of fast neutrons relative to 250 kVp X-rays, an RBE value of ^{60}Co gamma rays equal to 0·85 was taken into account.

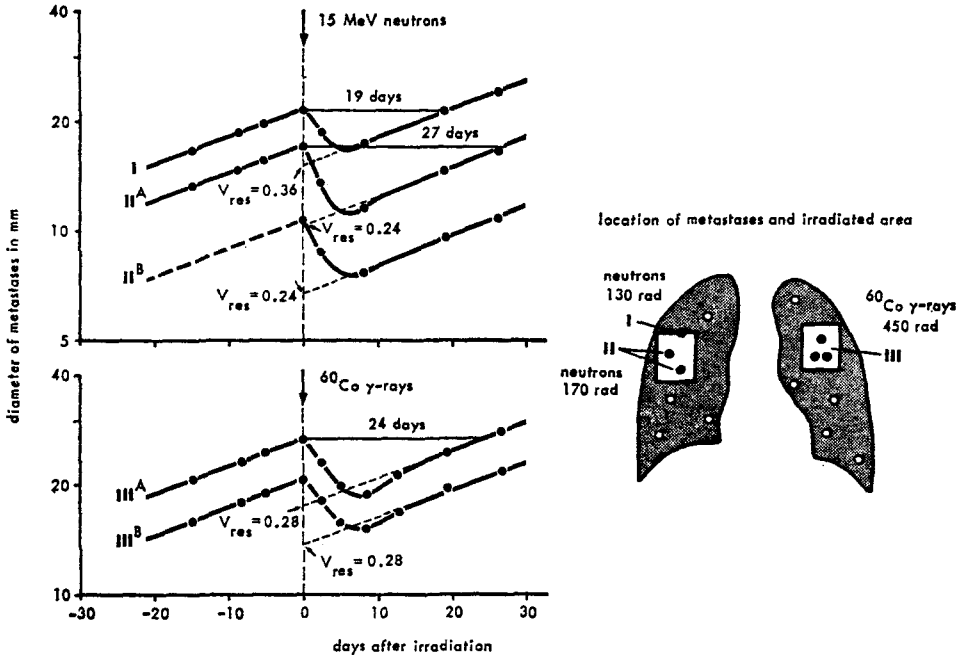


Fig. 1. Variation of the diameters of metastases in a patient with an embryonal carcinoma of the testicle (T_d=13 days) after irradiation with 15 MeV neutrons or gamma rays from ^{60}Co. The values of the growth delay, T_{GD}, are indicated as well as the extrapolated residual volume, V_{res}.

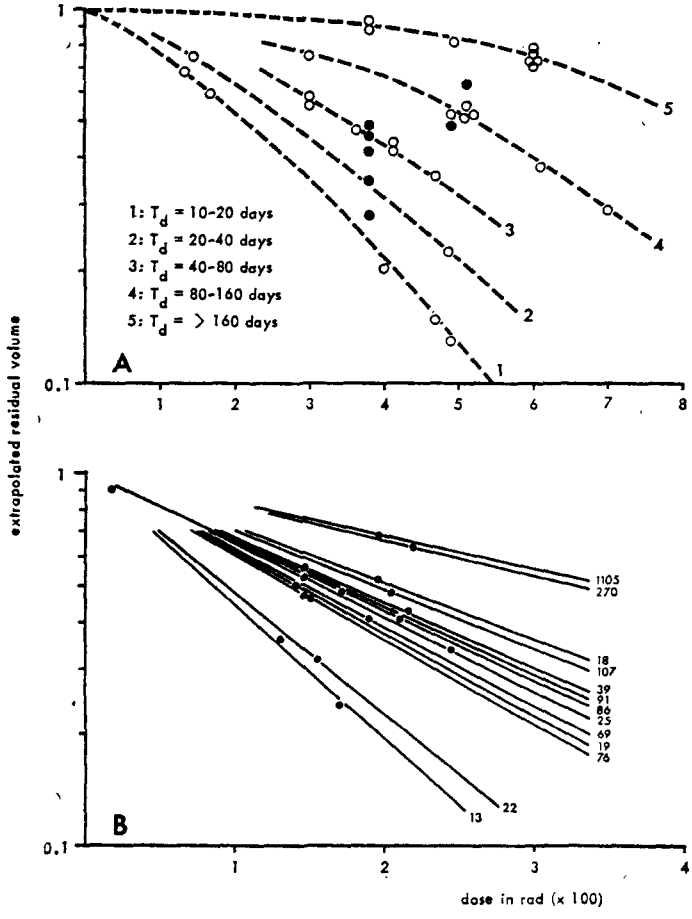


Fig. 2. Extrapolated residual volumes of pulmonary metastases of different tumours as a function of doses of 250 kVp X-rays (panel A) or 15 MeV neutrons (panel B). Doses of ^{60}Co gamma rays have been multiplied by 0·85 for comparison with 250 kVp X-rays. Curves 1, 2, 3, 4, and 5 represent average values for T_d=10–20 days, T_d=20–40 days, T_d=40–80 days, T_d=80–160 days and T_d>160 days, respectively. In the case of neutrons T_d values are shown individually.

RESULTS

An example of the data obtained is given in Fig. 1. In this case several metastases of an embryonal carcinoma of the testicle were present in both lungs. Within the limits of experimental accuracy they all had a volume doubling time $T_d = 13$ days. The doses administered were chosen to obtain a sufficiently large volume decrease of the metastases, while they were not so large as to interfere greatly with subsequent fractionated treatments with photons. As shown in Fig. 1, two metastases received 170 rad of neutrons, while a metastasis at the border of the field received 130 rad. Growth delay (T_{GD}) after 170 rad was 27 days corresponding to an extrapolated residual volume relative to the volume at the time of irradiation of $V_{res} = 0{\cdot}24$. After 130 rad these values were $T_{GD} = 19$ days and $V_{res} = 0{\cdot}36$. After 450 rad of ^{60}Co gamma rays, $T_{GD} = 24$ days and $V_{res} = 0{\cdot}28$ for both metastases measured.

In this patient, after a period of volume decrease and a short period of slightly accelerated regrowth, all metastases subsequently grew with a volume doubling time which was not significantly different from the T_d before irradiation. Furthermore, the data were obtained over a sufficiently long time interval after irradiation. In other cases a smaller number of radiographs was obtained and the extrapolated residual volume (V_{res}) was calculated assuming that the growth rate had reached the pre-irradiation value at the time at which the volume had returned to its pre-irradiation value. A summary of the values obtained from 13 patients is given in Table 1.

In Fig. 2, values of the extrapolated residual volume (V_{res}) from Table 1 have been plotted as a function of the dose. In Fig. 2A, seven solid points represent data obtained in the present series irradiated with ^{60}Co gamma rays, the 28 open circles represent data obtained previously with 250 kVp X-rays [5]. As mentioned earlier, these data indicate that the effectiveness per unit dose of photons decreases with increasing T_d of the metastases [3, 5]. As an approximation, curves have therefore been drawn, representing relationships between V_{res} and the dose for groups of tumours with T_d

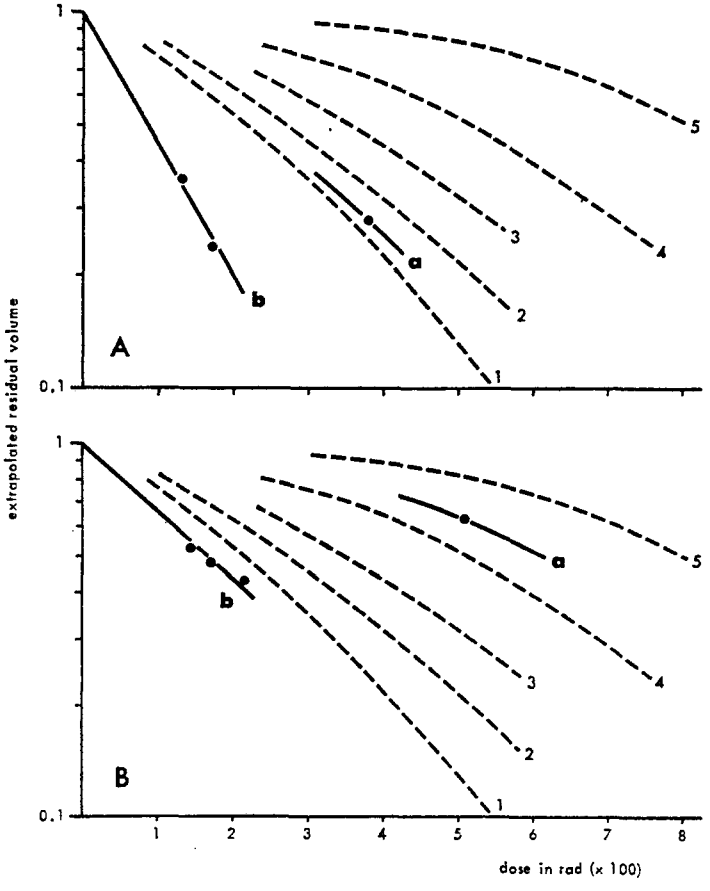


Fig. 3. Extrapolated residual volumes of pulmonary metastases as a function of doses of 250 kVp X-rays (curves a) or 15 MeV neutrons (curves b). Curves 1–5 are the average curves for 250 kVp X-rays as given in Fig. 2A. Panel A: $T_d = 13$ days; panel B: $T_d = 91$ days.

Table 1. Data concerning growth and radiation-induced growth delay of pulmonary metastases in patients

Properties of the metastases			15 MeV neutron irradiation			^{60}Co gamma irradiation				RBE	
Patient No.	Hist. diagnosis of the primary	T_d	Dose (rad)	Measured growth delay (days)	Extrapolated* residual volume	Dose (rad)	Dose† × 0·85 (rad)	Measured growth delay (days)	Extrapolated* residual volume	At neutron dose given	At neutron dose of 150 rad
1.	Embryonal ca. of the testicle	13	170	27	0·24	450	380	24	0·28	2·4	2·5
	Embryonal ca. of the testicle	13	130	19	0·36	450	380	24	0·28	2·5	2·5
2.	Anaplastic thyroid ca.	18	195	17	0·52	—	—	—	—	1·1	1·2
3.	Anaplastic thyroid ca.	19	140	19	0·50	450	380	24	0·42	2·2	2·2
4.	Squamous cell ca. floor of the mouth	22	155	36	0·32	—	—	—	—	2·5	2·6
5.	Reticula sarcoma of the skeleton	25	245	39	0·34	450	380	38	0·35	1·6	1·8
6.	Adeno ca. of the large bowel	39	145	33	0·56	450	380	40	0·49	2·3	2·3
7.	Osteosarcoma	69	190	90	0·41	—	—	—	—	2·2	2·4
8.	Adeno ca. of the large bowel	76	145	83	0·47	450	380	86	0·46	2·5	2·6
	Adeno ca. of the large bowel	76	150	84	0·46	—	—	—	—	2·5	2·6
9.	Squamous cell ca. of the bronchus	86	210	122	0·41	575	490	88	0·49	2·6	3·0
	Squamous cell ca. the bronchus	86	17	14	0·90	—	—	—	—	—	—
10.	Adeno ca. of the large bowel	91	215	110	0·43	600	510	60	0·63	3·1	4·0
	Adeno ca. of the large bowel	91	170	96	0·48	600	510	60	0·63	3·7	4·0
	Adeno ca. of the large bowel	91	145	84	0·53	—	—	—	—	4·1	4·0
11.	Squamous ca. of the bronchus	107	205	112	0·48	—	—	—	—	2·6	3·0
12.	Terato ca. of the testicle	270	220	172	0·64	—	—	—	—	3·2	4·0
13.	Chondro sarcoma	1105	195	608	0·68	—	—	—	—	3·4	3·9

*Relative to volume at time of irradiation.

†Dose × RBE of ^{60}Co gamma rays relative to 250 kVp X-rays. This value is used to derive RBE values of neutrons relative to 250 kVp X-rays.

values of 10–20, 20–40, 40–80, 80–160, and more than 160 days, respectively. The data obtained with 15 MeV neutrons have been plotted in Fig. 2B. Where data are available for two doses, they are not inconsistent with exponential relationships and therefore all curves are drawn as exponential curves.

In order to derive RBE values, curves obtained for 15 MeV neutrons and for gamma rays were compared. Two examples are shown in Figs. 3A and B. In Fig. 3A the data derived from the growth curves of Fig. 1 are presented. Curve a is drawn through the point for gamma rays with a slope parallel to curve 1, curve b is drawn as an exponential through the points obtained with 15 MeV neutrons. Comparison of the point at 130 rad of neutrons with the corresponding point of curve a at 320 rad yields an RBE = 2·46 and comparison of the point at 170 rad with the corresponding point of curve a at 415 rad yields an RBE value of 2·44. These values are given as 2·5 and 2·4, respectively, in Table 1. The RBE at 150 rad of neutrons derived from curves a and b is equal to 2·5. This value is given in the last column of Table 1. In Fig. 3B data have been plotted obtained from an adenocarcinoma with a $T_d = 91$ days. Curve a through the point for gamma rays is drawn parallel to curve 4. The RBE values derived from the comparison of curves a and b are given in Table 1. In this Table all other RBE values are also presented. For some cases the effect of a dose of ^{60}Co gamma rays could not be evaluated because

the metastases developed blurred contours. In order to derive RBE values in these cases, the neutron data were compared with the average curve for gamma rays for the group with corresponding T_d's, as presented in Fig. 2.

DISCUSSION

In all cases the doses administered were large enough to give a considerable reduction in volume, while they were not so large as to interfere with subsequent conventional radiotherapy. In most cases a period of accelerated growth was observed shortly after the minimum volume had been attained. None of the patients showed any effect on their skin.

In a few cases the same metastasis could be irradiated with gamma rays and subsequently with neutrons, while in the same patient another metastasis received neutrons first and gamma rays later. No difference in RBE value due to this difference in sequence was observed.

The data of Fig. 2 show a considerable variation in the effectiveness per unit dose for photons as well as for neutrons. For instance, the doses required to obtain 50% reduction in extrapolated residual volume range from 210 to 850 rad for photons and for neutrons from 85 to 350 rad. It is of interest to note, however, that if the extremes are left out, for fast neutrons in 70% of the cases, the range of doses required for 50% reduction is quite small (130–210 rad), while for photons the corresponding range is 250–600 rad. This indicates that the spread in sensitivity for the majority of the metastases is much larger for photons than for neutrons, although exceptions are evident.

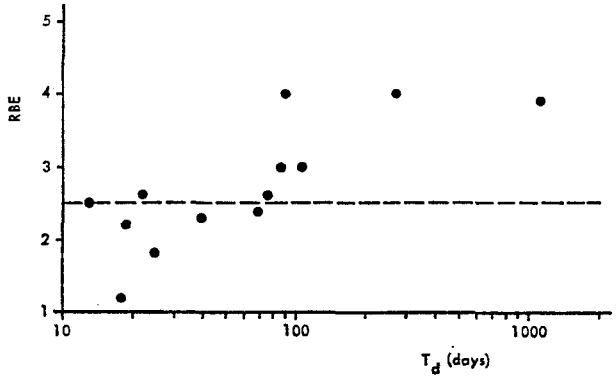


Fig. 4. RBE values of 15 MeV neutrons relative to 250 kVp X-rays for volume reduction of pulmonary metastases in patients as a function of the volume doubling time.

In Fig. 4 the RBE values derived for volume reduction of lung metastases at a dose of 150 rad of 15 MeV neutrons are presented as a function of the volume doubling time T_d. It can be deduced that in general larger RBE values are observed for large T_d values. For damage to skin and intestine of rats and mice, RBE values at a dose per fraction of 150 rad of 15 MeV neutrons are in the range of 2·3–2·7 [7]. The mean value of 2·5 is indicated by the dotted line in Fig. 4. If the same value would be applicable for human normal tissue tolerance, it could be concluded that 15 MeV neutrons should provide an advantage only for treatments of tumours with T_d values in excess of about 80 days. No RBE values are yet available for 15 MeV neutrons with respect to tolerance of dose-limiting normal tissues in patients. However, it is unlikely that these normal tissue RBE values depend on the growth rate of tumours growing adjacent to these tissues. This implies that a significant gain from the use of fast neutrons is most likely to be obtained for slow growing tumours. Further data are required, however, for primary tumours and fractionated treatments to establish the conclusion firmly.

REFERENCES

1. K. Breur and H. A. van Peperzeel, Possible clinical models for quantitative comparison of effects of different radiations on tumours. *Europ. J. Cancer* **7,** 199 (1971).
2. K. Breur, Growth rate and radiosensitivity of human tumours—I. Growth rate of human tumours. *Europ. J. Cancer* **2,** 157 (1966).
3. K. Breur, Growth rate and radiosensitivity of human tumours—II. Radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 173 (1966).
4. G. W. Barendsen, J. J. Broerse, K. Breur and H. A. van Peperzeel, Evaluation of possible advantages of 15 MeV neutrons for tumour radiotherapy. *TNO–Nieuws* **27,** 721 (1972).
5. H. A. van Peperzeel, Effects of single doses of radiation on lung metastases in men and experimental animals. *Europ. J. Cancer* **8,** 665 (1972).

6. J. J. BROERSE, J. E. BROERS-CHALLISS and T. MARUYAMA, Dosimetry of D–T neutrons for radiotherapeutic applications. *Proc.* 1*st Symp. Neutron Dosimetry in Biology and Medicine*, Munich (Edited by G. BURGER, H. SCHRAUBE and H. G. EBERT), p. 1. Commission of the European Communities, Luxembourg (1972).

7. G. W. BARENDSEN and J. J. BROERSE, Relative biological effectiveness of fast neutrons for effects on normal tissues. In *Current Topics in Radiation Research Quarterly* (Edited by M. EBERT and A. HOWARD), Vol. 8, p. 305. North-Holland, Amsterdam (1973).

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Clinical Features of 16 and 50 $MeV_{d\rightarrow Be}$ Neutrons

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Abstract—*The clinical trial of* 16 *and* 50 $MeV_{d\rightarrow Be}$ *neutron therapy is discussed and a comparison of the neutron beams and an outline of the development of dosage schedules are given.*

INTRODUCTION

IN OCTOBER 1972, M. D. Anderson Hospital initiated a clinical trial of fast neutron therapy using the Texas A & M Variable Energy Cyclotron, an 88-in. multiparticle machine capable of accelerating deuterons to energies from 12 to 60 MeV. Initially, the study used neutrons produced by bombarding beryllium with 16 MeV deuterons since there was clinical experience at Hammersmith with this energy. Since March 1973, 50 MeV deuterons have been employed.

The objectives of the present paper are: (1) to compare 16 and 50 MeV neutron beams with regard to physical parameters of clinical importance, i.e. skin sparing, depth dose, and isodose distribution; and (2) to outline the development of dosage schedules.

PHYSICAL PARAMETERS

Dosage determination

Energy designations refer to the energy of the incident deuterons. The doses have been calculated using measurements obtained with a tissue-equivalent (TE) ionization chamber immersed in TE liquid. They are expressed in rad including both neutron and gamma components ($rad_{n\gamma}$), because neutron cross sections are not adequately known for energies above 14 MeV; consequently it is impossible to accurately calculate the neutron response of gamma detectors. The gamma contribution may be as much as 3–9% of the total dose at the surface and 12–14% at 15 cm depth [1].

Skin sparing

With the 16 MeV beam, the surface dose is 30% of the maximum build-up which occurs at 2·0 mm depth; whereas with the 50 MeV beam, the surface dose is 43% of the maximum build-up which occurs at 8·0 mm (Fig. 1). Skin reactions with the 16 MeV beam are comparable to those seen with ^{137}Cs γ-rays. With the 50 MeV beam, acute skin reactions are similar to those seen with ^{60}Co because the doses are equivalent at the level of the stratum germinativum (0·5–1·0 mm).

Depth dose

With a 10×10 cm field, the depth dose at 10 cm is 41·5% of the maximum dose with the 16 MeV beam (126·5 cm SSD) and 63·8% of the maximum dose with the 50 MeV beam (140 cm SSD) (Fig. 1). Deep-seated tumours are easily accessible to the 50 MeV beam. Three patients with carcinoma of the esophagus and four with pelvic tumours have been irradiated with 1800–2000 $rad_{n\gamma}/5\frac{1}{2}$–7 weeks with only mild erythematous skin reactions.

Isodose distributions

With the 16 MeV beam, the 60% isodose curve is flat to within 10% of the axis dose

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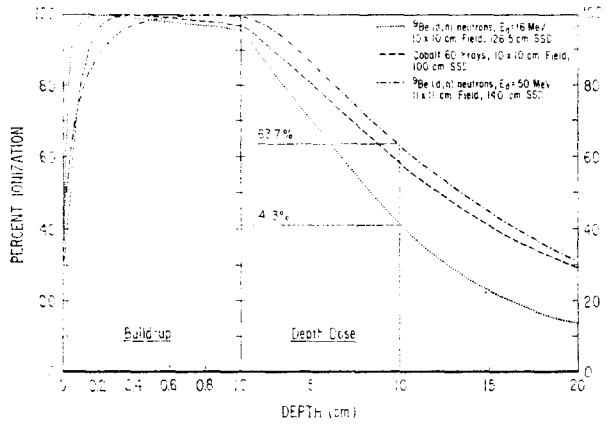


Fig. 1. Surface build-up and depth–dose curves for 16 and 50 MeV $_{d\rightarrow Be}$ neutrons and ^{60}Co γ-rays.

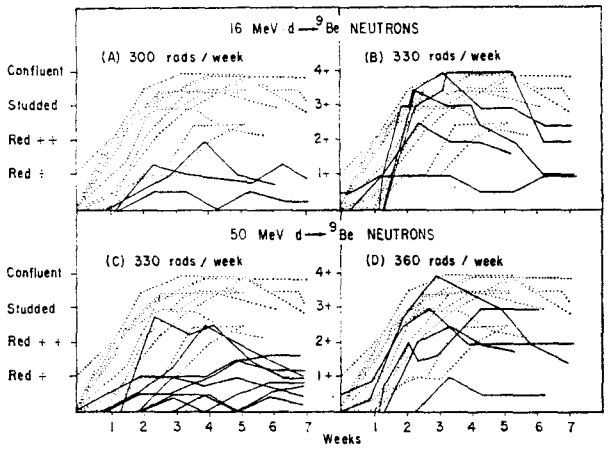


Fig. 2. Mucosal reaction patterns in 21 patients treated twice weekly with the 16 or 50 MeV beams. The solid lines represent the mucosal patterns seen with neutrons (A—16 MeV, 300 $rad_{n\gamma}$/week; B—16 MeV, 330 $rad_{n\gamma}$/week; C—50 MeV, 330 $rad_{n\gamma}$/week; D—50 MeV, 360 $rad_{n\gamma}$/week). The dotted lines represent the reactions produced with ^{60}Co at doses of 1000 rads/5 Fx/week.

Mucosal reaction grades: (1+) mild erythema, (2+) brisk erythema, (3+) studded fibrinous mucositis, (4+) confluent fibrinous mucositis.

over 70% of the designated field size; with the 50 MeV unfiltered beam, the 60% isodose curve is flat to within 10% of the axis dose over only 48% of the field. This can be improved with a beam flattening filter to produce a homogeneous dose over greater than 90% of the designated field size.

EVOLUTION OF DOSAGE SCHEDULES

Clinical equivalence

Mucosal reactions were used to evaluate equivalence with ^{60}Co γ-rays since a 10% change in dosage produces a clinically detectable difference in mucosal reactions [2]. Observations of skin reactions are not accurate to 10% [3, 4]. The majority of patients treated with ^{60}Co γ-rays at doses of 1000 rads/5 fractions (*Fx*)/week develop a studded or confluent fibrinous mucositis 12–17 days after the beginning of treatment (Fig. 2).

The initial assumption was that 1000 rads/5 *Fx* per week is equivalent to 800 rads/2 *Fx* per week based on NSD calculations. Our pretherapeutic studies had shown that the RBE's for this fraction size were 2·65 for the 16 MeV beam and 2·4 for the 50 MeV beam. Initially equivalences were derived as follows:

1. Standard treatment at MDAH: 1000 rads/5 *Fx* per week (^{60}Co γ-rays).
2. Equivalent to: 800 rads/2 *Fx* per week (^{60}Co γ-rays).
3. Equivalent to: 300 $rad_{n\gamma}$/2 *Fx* per week (16 MeV; RBE 2·65).
   or: 330 $rad_{n\gamma}$/2 *Fx* per week (50 MeV; RBE 2·4).

The first three patients treated with the 16 MeV beam received 300 $rad_{n\gamma}$ per week (Fig. 2A). None developed the studded or the confluent mucositis seen with ^{60}Co at 1000 rads/5 *Fx* per week. Two of these showed continued growth of tumour (each histologically confirmed at 3 months). With an increase to 330 $rad_{n\gamma}$, subsequent patients developed a studded to confluent mucositis (Fig. 2B). When treatment with the 50 MeV beam was initiated, 330 $rad_{n\gamma}$ per week were first employed. The first eight patients developed only a mild erythema (Fig. 2C), but an increase to 360 $rad_{n\gamma}$ resulted in a studded to confluent mucositis (Fig. 2D).

Because the lower dosage schedules failed to produce the expected reaction pattern, we tested whether this difference was due to an error in RBE determination or an incorrect assumption that 1000 rads/5 *Fx* per week with ^{60}Co γ-rays is equivalent to 800 rads/2 *Fx* per week. Seven patients were treated with ^{60}Co γ-rays giving 400 rads twice weekly through parallel opposing portals covering the oropharynx and faucial arch. This produced only a mild erythema similar to that produced by 900 rads/5 *Fx* per week with ^{60}Co, or by 300 $rad_{n\gamma}$/2 *Fx* per week and 330 $rad_{n\gamma}$/2 *Fx* per week with 16 and 50 MeV neutrons, respectively.

Until the equivalent ^{60}Co twice weekly dose is known, RBE's for mucosal reactions cannot be calculated. A preferred expression is the

Mucosal Equivalence Factor (M.E.F.), which incorporates both the RBE and the fractionation factor:

$$\text{M.E.F.}_{16\text{ MeV}} = \frac{1000\text{ rads/week}}{330\text{ rad}_{n\gamma}\text{/week}} = 3{\cdot}0$$

$$\text{M.E.F.}_{50\text{ MeV}} = \frac{1000\text{ rads/week}}{360\text{ rad}_{n\gamma}\text{/week}} = 2{\cdot}8$$

Clinical planning

The dosage schedules have been designed to deliver the equivalent of 1000 rads of ^{60}Co γ-rays per week to a level of 7000 rads which has been the practice at MDAH for advanced tumours [5]. Mucosal reactions are easily monitored and shrinking field techniques can be used.

1. For elective irradiation of subclinical disease, an integral part of any treatment, 5000 rads in 5 weeks with ^{60}Co γ-rays are always used since there is no anoxic component assumed.

2. For the irradiation of gross masses, two approaches have been employed:

(*a*) Entire irradiation with neutrons: this course is selected when both the primary and regional areas contain bulky disease requiring large fields throughout the treatment.

(*b*) Boost irradiation with neutrons: this plan is selected when a significant field size reduction is feasible for boosting the dose to palpable disease. After 5000 rads in 5 weeks with ^{60}Co γ-rays to the gross tumour and noninvolved regional lymphatics, a neutron boost equivalent to 2000 rads [165 $\text{rad}_{n\gamma} \times 4$ (16 MeV), or 180 $\text{rad}_{n\gamma} \times 4$ (50 MeV)] is given to palpable disease.

Clinical material

Between October 1972 and September 1973, 72 patients were treated; 34 patients with head and neck cancer, 11 patients with breast carcinoma, 8 with superior sulcus bronchogenic carcinoma, and 19 with a variety of miscellaneous neoplasms. All had far advanced cancer. Very few were excluded because of age, general medical condition, or debility. None were excluded because the disease was so far advanced that they were deemed incurable. The 16 MeV beam was used in 27 patients, 50 MeV in 38, and both in 7.

The preliminary results are outlined in Table 1. The local control and complication rates are subject to change since recently treated patients may show further regression, and further complications and recurrences might still develop. A preliminary evaluation indicates that the local control rate in head and neck and breast carcinomas is high (24/34 for head and neck carcinomas, 8/11 for breast carcinomas). However, the complication rate is high, particularly in patients with extensive primary head and neck tumours.

Discussion

A pretherapeutic study at TAMVEC [6] showed that acute mucosal reactions with neutrons did not correlate with late changes. Five Rhesus monkeys were irradiated with the 16 MeV beam (167 $\text{rad}_{n\gamma} \times 13/6\frac{1}{2}$ weeks), five with 50 MeV (170 $\text{rad}_{n\gamma} \times 13/6\frac{1}{2}$ weeks), and

Table 1. *Preliminary results* (72 *Patients—October*, 1972–*September*, 1973)

	Neutron beam			Complications (exclud. fibrosis, xerostomia)	Local control (3–12 mo)	Survival (2–12 mo)
	16 MeV	50 MeV	Both			
Head and neck cancer						
Extensive 1° disease	8	11	3	8/22	16/22*	19/22
Extensive neck disease	3	6	3	2/12	8/12	10/12
	11	17	6	10/34	24/34	29/34
Breast carcinoma	4	7		0/11	8/11†	9/11
Superior sulcus carcinoma	6	2		0/8	5/8	5/8
Miscellaneous	6	12	1	3/19	7/19	14/19
Total	27	38	7	13/72‡	44/72	57/72

*One patient scored as a local failure is NED as a result of surgical salvage.

† One patient, treated palliatively, was scored as a local failure due to marginal recurrence.

‡ 2 exposed mandibles; 2 mucosal necrosis; 3 skin necrosis; 3 massive haemorrhage; 1 laryngeal oedema; and 2 massive necrosis and/or tumour.

five with ^{60}Co γ-rays (200 rads × 33/6½ weeks). Although acute reactions were similar in all three groups, the late effects were more severe in the 16 MeV irradiated group. All five animals in this group developed osteoradionecrosis; whereas, the monkeys irradiated with ^{60}Co γ-rays, and thus far those irradiated with the 50 MeV beam, have developed no significant late changes.

Despite equivalent mucosal reactions with ^{60}Co γ-rays, early impressions in the clinical trial suggest that the late effects with both 16 and 50 $MeV_{d \rightarrow Be}$ neutrons are more severe. Consequently, schedules are being reevaluated based on the extent of the local tumour and its anatomical location. Ultimate treatment schedules will be based on a balance of local control rates with an aceeptable incidence of severe complications and bothersome sequelae.

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REFERENCES

1. P. R. Almond, J. B. Smathers, G. D. Oliver, E. B. Hranitzky and K. Routt, Dosimetric properties of neutron beams produced by 16–60 MeV deuterons on beryllium. *Radiat. Res.* **54,** 24 (1973).
2. G. H. Fletcher, W. S. MacComb and R. J. Shalek, *Radiation Therapy in the Management of Cancer of the Oral Cavity and Oropharynx*, Charles C. Thomas, Springfield, Ill. (1962).
3. G. H. Fletcher and H. T. Barkley, Jr., Present status of the time factor in clinical radiotherapy. Part 1. The historical background of the recovery exponents, *J. Radiol.* (In press).
4. G. H. Fletcher., H. T. Barkley, Jr., L. J. Shukovsky, Present status of the time factor in clinical radiotherapy. Part 2. The nominal standard dose formula. *J. Radiol.* (In press).
5. G. H. Fletcher, *Textbook of Radiotherapy*. Lea & Febiger, Philadelphia, 2nd Edn. (1973).
6. J. H. Jardine, D. D. Boyd, D. H. Hussey, T. J. Davidson and T. E. Daly, Oral cavity irradiation effects in Rhesus monkeys: a comparison of 16 and 50 MeV neutrons and ^{60}Co (In preparation).

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Comparison between Neutron Therapy and ^{60}Co Gamma Ray Therapy of Bronchial, Gastric and Oesophagus Carcinomata

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Abstract—*In order to study qualitative differences in effectiveness between radiation of low LET and of high LET on human tumours* in vivo, *results from* 147 *cases of bronchial carcinoma which as a control series had been irradiated with telecobalt therapy only, have been compared with results from a series of* 20 *cases receiving as part of their treatment, cyclotron produced neutrons in a proportion of the total dose of* 20% *and with results from* 15 *cases receiving a* 35% *proportion of neutrons. A common standard was employed by histological examination of all cases (autopsy findings and surgical specimens) for surviving tumour rests. Smaller control series of oesophagus and gastric carcinomata were studied in the same way. All series exhibited a distinctly stronger effectiveness of the combined irradiations with neutrons and gamma rays, which for the bronchial carcinomata treated with a* 35% *proportion of neutrons was confirmed statistically.*

The statistical evaluation of the comparability of the series (tumour size, total dose, total irradiation time, histological tumour types) corroborated the findings concerning the qualitatively higher neutron effectiveness in all respects. The difference in sequences of the irradiation, i.e. N–γ or γ–N, as well as the length of the intervals did not cause observable changes in effectiveness.

INTRODUCTION

FOLLOWING the first reports from Hammersmith Hospital, London, we started studies of neutron therapy in April 1972, using the Soviet cyclotron of the type U 120, which is available in the GDR. As a cooperative project of several institutes of the Academy of Sciences and the Medical Academy of Dresden, the apparatus which is normally used for fundamental research, was equipped for the irradiation of patients and the necessary dosimetry was carried out.

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The cyclotron is located at 250 km distance from our Institute and is not attached to a hospital. Therefore, we can use the apparatus only during selected periods. Up to now, 175 patients have been irradiated. At the German and Austrian Congress of Radiology in 1973 we reported results of a first series of patients. The data showed a distinctly higher effectiveness of combined neutron + telecobalt irradiation on different tumours as compared with exclusive telecobalt therapy [1]. In this paper results from a larger number of patients are reported.

AIM AND METHODS

The first aim of our studies was to gain insight into possible qualitative differences in effectiveness between neutron radiation and ^{60}Co gamma radiation for effects on tumours *in vivo*. In all cases the analyses depend on histological examination of the total irradiated area in the surgical or autopsy specimens, to investigate the presence of surviving tumour rests. A number of preceding series of tests with telecobalt therapy only, provided us with material for comparison and with established histological examination methods [2]. The criterion "tumour histologically no longer detectable" means that, after thorough macroscopic selection, many microscopic sections were made, revealing only cicatricial tissue and necrosis but no tumour cells. This does not prove the complete destruction of all tumour cells, because this would require a very large number of serial sections, but it provides a fairly constant standard of tumour cell reduction.

We applied a total tumour dose comprizing only a fraction of between 20–35% neutron radiation to the majority of patients. This choice was made for two reasons:

1. Since in the case of our cyclotron the depth dose characteristics are unfavourable as compared with that of ^{60}Co gamma radiation, deep-seated tumours would require a four-portal technique. We were, however, reluctant to use the high volume dose with neutron radiation, because of uncertainties about the RBE for small doses in various tissues and about volume factors. Therefore, irradiation was carried out with the two opposed-portals technique.

2. Anoxic conditions of cells, for which an advantage from neutron radiation is expected, are assumed to prevail only for small areas in the tumours. After partial destruction of cells in these areas, reoxygenation should become possible, resulting in turn in a normal sensitivity to ^{60}Co gamma radiation. If this reoxygenation hypothesis is correct for fractionated radiation therapy, application of fast neutrons as a fraction of the total dose should be sufficient.

In order to derive a measure of the total tumour dose resulting from combined neutron and ^{60}Co gamma therapy, the neutron dose was converted into gamma ray equivalent units by means of an RBE curve based on data given in literature and obtained from own studies [3, 4]. From these data we derived RBE values from 3·5 to 2·6 for neutron dose fractions ranging from 40 up to 90 rad. All irradiations were fractionated five times a week. For combined series we introduced an interval between the neutron and the telecobalt therapy, sometimes 3–8 days, sometimes 18–30 days, to allow for a possible reoxygenation. It should be noted that only half of the cases in the control series with exclusive telecobalt therapy has been irradiated with this type of "split course schedule". These latter cases, however, did not exhibit any difference in effectiveness compared to those cases which had been irradiated without an interval.

RESULTS

Our results are summarized in Table 1. For histologically proven bronchial carcinoma a comparison of the radiation effect is given between 149 cases which had been treated exclusively with telecobalt therapy and a number of cases which had received a neutron dose proportion: 20 cases with 20% (in gamma ray equivalent units) and 15 cases with 35%. The stronger effect of the combined irradiation (35% neutrons) as shown by 11 cases of complete tumour destruction in a total of 15 cases, has been statistically confirmed (χ^2 test, 99% level). Furthermore, a comparison is presented between 6 cases of gastric carcinoma treated exclusively with telecobalt therapy and 7 cases which received a combined irradiation (30% neutron dose proportion). After exclusive telecobalt therapy considerable tumour tissue was still present, whereas after combined therapy 3 out of 7 tumours were completely destroyed, and the other cases manifested only dispersed tumour islets. Finally, 65 cases with squamous epithelioma of the oesophagus, treated exclusively with telecobalt therapy, are compared with 11 cases of combined therapy with a neutron dose proportion of 27%. Again the combined therapy which led to disappearance of tumour in 6 out of 11 cases appears to be superior to exclusive telecobalt therapy which yielded 25 destructions in 65 cases.

DISCUSSION

In order to examine the comparability of the bronchial carcinoma series, all additional factors which can influence the results were statistically evaluated (Table 1).

The tumour sizes were calculated from measurements of tumour diameters on special X-ray photographs for two perpendicular planes assuming the shape of a rotation ellipsoid [2]. It can be seen that the average tumour size in the series with the strongest radiation

Table 1. Results of tumour treatments with neutron + gamma radiation in comparison with gamma radiation only

Number of patients	Tumour dose			Tumour volume (cm^3)	Total time (days)	Analysis for tumour rests (negative)	
	γ rays (R)	Neutrons (rad)	Total* (eq. units)			Number	Fraction of total
Bronchial carcinoma							
149	7044	—	7044	212	52	47	0·33
	(904)†	—	(904)	(145)	(12)		
20	4604	393	5657	168	63	10	0·50
	(465)	(34)	(452)	(70)	(17)		
15	3236	706	5226	214	64	11	0·74
	(651)	(90)	(677)	(80)	(11)		
31		762	2175	216	24	2	0·66
		(39)	(74)	(106)	(0)		
Stomach carcinoma							
6	5233	—	5233	—	44	0	0
	(1082)	—	(1082)	—	(31)		
7	3250	586	4880	—	65	3	0·42
	(919)	(188)	(145)	—	(11)		
Oesophagus carcinoma							
65	8203	—	8203	8·5‡	68	25	0·39
	(865)	—	(865)	(2·6)	(28)		
11	4350	503	5548	9·1‡	65	6	0·55
	(696)	(192)	(655)	(2·0)	(15)		

*Gamma ray equivalent units = gamma ray dose + neutrons dose × RBE for the given dose per fraction.
†In parentheses the standard deviations for variation between patients.
‡One dimension only given in cm.

effect (35% neutrons) is not different from that in the control series. An analysis of individual cases showed that the complete destruction of small tumours was obtained as frequently as that of large ones. The average irradiation time of the two neutron series was 20% longer than that of the control series, whereas the total dose (in gamma ray equivalent units) is lower by 20% and 25%, respectively. Hence, the evaluation of all these parameters of the radiation effect confirmed the conclusion of a stronger effect on the tumour induced by the combined neutron + gamma ray irradiation.

The study of the differentiation stage of the tumours revealed 62% (94/149) well differentiated tumours in the control series (squamous and adenocarcinoma), but only 46% (7/15) in the series with large neutron dose proportion, and 75% (15/20) in the series with small neutron dose proportion. Calculation of the fraction of complete destructions for all undifferentiated tumours (undifferentiated carcinoma, oat cell carcinoma), in the control series. It amounted to 19 out of 51 = 0·37. This fraction is still considerably smaller than that for the combined neutron + gamma ray irradiation.

The interval between the neutron and the telecobalt irradiation (3–8 and 18–30 days, respectively) was of no consequence for the irradiation result. For both combined series it was found that complete tumour destruction was effected after a short interval as often as after a long one. Two patients received the neutron irradiation (35%) at the end of the irradiation; in both cases the tumour was destroyed. In five cases the interposed neutron dose was followed by a gamma proportion of 15% of the total dose. This led also to complete tumour destruction for 4 out of 5 cases. Hence, a reoxygenation phenomenon, at least in the sense of recapillarization, is apparently not necessary for the increased effectiveness of neutron irradiation.

The conclusions derived from the comparison of exclusive telecobalt treatments, and of the combined neutron–telecobalt series were, in principle, confirmed for the two other tumour types (stomach, oesophagus).

It can be concluded that all our results obtained so far indicate a stronger effectiveness of the combined radiations on the tumour types under study. In addition, a neutron proportion of 35% seems to be more effective than that of 20%. Finally our results indicate that the sequence in which neutrons and gamma rays are given is not important. The same is true for the duration of the interval between the irradiation proportions with low and with high LET, respectively.

REFERENCES

1. H. -J. Eichhorn and A. Lessel, Klinische Erfahrungen mit Neutronentherapie. *Arch. Geschwulstforsch.* **43,** 268 (1974).
2. H. -J. Eichhorn, A. Lessel and K. H. Rotte, Einfluss verschiedener Bestrahlungsrhythmen auf Tumor- und Normalgewebe *in vivo*. *Strahlenth.* **143,** 614 (1972).
3. 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).
4. E. Magdon, Relative Biologische wirkgamkeit (RBW) schneller Neutronen bei mensorlichen fellkulturen aus embryonalem und malignem gewebe. Fortschvill der onkologie, Academic-velag, Berlin 1974 (In press)

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The Use of Californium-252 Sources for Brachytherapy of Human Tumours: A Preliminary Report

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Abstract—*Various aspects of the use of californium-252 fission neutrons for treatments of tumours in patients are discussed, including dosimetry, radiation protection problems and the selection of patients suitable for initial applications. From early results an RBE value of 7·0 is suggested as a fairly close approximation of the efficiency of these neutrons in the mixed emission from ^{252}Cf at the dose rates used in clinical implantation of these sources.*

INTRODUCTION

THE ARGUMENTS for the use of fast neutrons in the radiotherapy of human tumours have already been outlined by other authors, (e.g. [1]). A compact neutron-emitting source which can be implanted into tissues for brachytherapy exactly as has been done previously with gamma-emitting sources [2] is provided by californium-252. Fifty Short Afterloading Cells (SALC) and thirty longer Afterloading Cells (ALC) containing ^{252}Cf were provided on loan by the U.S. Atomic Energy Commission.† The first implantation of SALC sources into a patient was performed eleven months after the arrival of the sources in Oxford in October 1971. To date, nine patients have had suitably accessible lesions implanted with these SALC sources. The longer ALC sources have proved too difficult to afterload into the patient and have therefore not been used clinically.

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†The californium-252 sources were loaned by the U.S. Atomic Energy Commission under contract number AT (38–1)–630 for a medical research and study programme.

DOSIMETRY

Non-uniformity of activity along the length of the longer ALC sources caused some concern, which led to replacement of a proportion of these sources; only a few of the SALC showed measurable radioactivity non-uniformities, but many had slightly enlarged ends which made them difficult to afterload, and these too were replaced by the U.S.A.E.C. Relative activities of the sources were measured using a BF_3 counter with the sources held in a polythene "castle"; these were compared with the absolute activity of one ^{252}Cf source which was measured in a Manganese bath at the National Physical Laboratory, Teddington. This measurement agreed to within 1% of the calibration provided by the U.S.A.E.C., when corrections were made for radioactive decay between the calibrations.

Source distribution and dosimetry has been based on the "Relational System" of Pierquin

[3]. Each implant has consisted of parallel active lines without crossed ends, generally separated by between one and two centimetres, in one or two planes. The reference point, or isodose, at which the tumour dose to be delivered was specified, was 0·5 cm distant from the plane of the sources, opposite a point midway between the central source lines, halfway along their length. Transverse tomography was used to determine the relative positions of the source lines and of the skin, for calculation purposes [2]. No adjustment of overall time was made for any supposed difference in radiobiological effect due to varying dose rate at this reference point.

The calculations of the doses delivered from these implants of ^{252}Cf in the clinical radiobiological studies described here have been made using the depth dose tables of Colvett *et al.* [4]. As the emission from ^{252}Cf consists of a mixture of neutrons and gamma rays, the planned dose in "effective rad" was taken as the neutron dose in rad × RBE, plus the gamma dose in rad. Complete isodose distributions have been calculated using the CALRAD computer programme, by courtesy of the Physics Department, M.D. Anderson Hospital and Tumor Institute, Houston, Texas [5].

The dose in "effective rad" which was delivered at the reference isodose varied in these patients between 6000 and 4250 rad. A reduction was made from the higher dose level in patients who had had previous radiation, especially where the reference isodose lay close to the skin, and an added reduction was used in the case of very aged patients.

SOURCE TRANSPORT AND SHIELDING: RADIATION PROTECTION

The storage safe for the ^{252}Cf sources in the isotope laboratory was fabricated from the transport containers in which the sources had been shipped from the U.S.A.: a workbench shielded with 20 cm of water protection was provided for the physics technician whilst loading the sources into thin plastic tubes from which they would be transferred to similar tubes implanted in the patient's tumour. The sources were transported from the isotope laboratory to the ward in a 50 cm diameter cylindrical container filled with borated (0·5% boron w/v) paraffin wax ($C_{30}H_{62}$). Personnel shields used by the radiotherapist afterloading the ^{252}Cf sources into the patient, and by nursing staff whilst the sources were in the patient, were also filled with the same borated paraffin wax. These shields had clear viewing areas provided by a perspex tank filled with a 25% solution of neutralised boric acid (4% boron w/v) maintained in solution in 15% dextrose; this solution was optically clear. The fluid-filled tank was considerably cheaper than the thick perspex blocks which have been used at other centres [5]. The use of this shield is shown in Fig. 1(*a*). The technique of transferring the ^{252}Cf sources from thin plastic tubes into similar tubes already implanted into the patient was evolved from the technique used for afterloading ^{192}Ir gamma sources for brachytherapy [2]. The complete, loaded implant is shown in Fig. 1(*b*). A tissue-equivalent neutron counter was constructed and used to make measurements of environmental dose rates on every occasion that ^{252}Cf sources were implanted into patients. Working behind the personnel shield the dose to the radiotherapist was less than 1 mrem per implant. On average, the dose-rate at the same position unshielded was *ca.* 30 mrem/hr, and the dose-rate at the foot of the bed with no shielding other than that provided by the patient was *ca.* 5 mrem/hr.

CLINICAL RADIOBIOLOGY

It was agreed that the ^{252}Cf sources would be used in the first instance for implantation into superficial or accessible tumours either in patients who had disseminated or locally recurrent disease. However, the patients were expected to have a sufficiently good prognosis to allow follow-up for periods of several months or longer. Measurements of the size of the tumour were to be made at the time of implantation, weekly for one month, fortnightly for another two months, and thereafter at monthly intervals. Normal tissue response would be observed in the skin overlying the implanted area, using a quantitative numerical scale [6].

The data for the nine patients into whom we have so far implanted ^{252}Cf sources is summarised in Table 1. For the first three patients, an RBE of 7·5 was used for the neutron component of the dose from ^{252}Cf. As a precautionary measure this RBE value was somewhat higher than that extrapolated from radiobiological data [7, 8] and the preliminary clinical experience at the M. D. Anderson Hospital [5]. In patient No. 2 (AC), who had multiple skin metatases, an opportunity arose to compare directly the effects of ^{252}Cf and ^{192}Ir implants in tumours of similar size and location in the same patient. There was less early skin reaction from the ^{252}Cf implant,

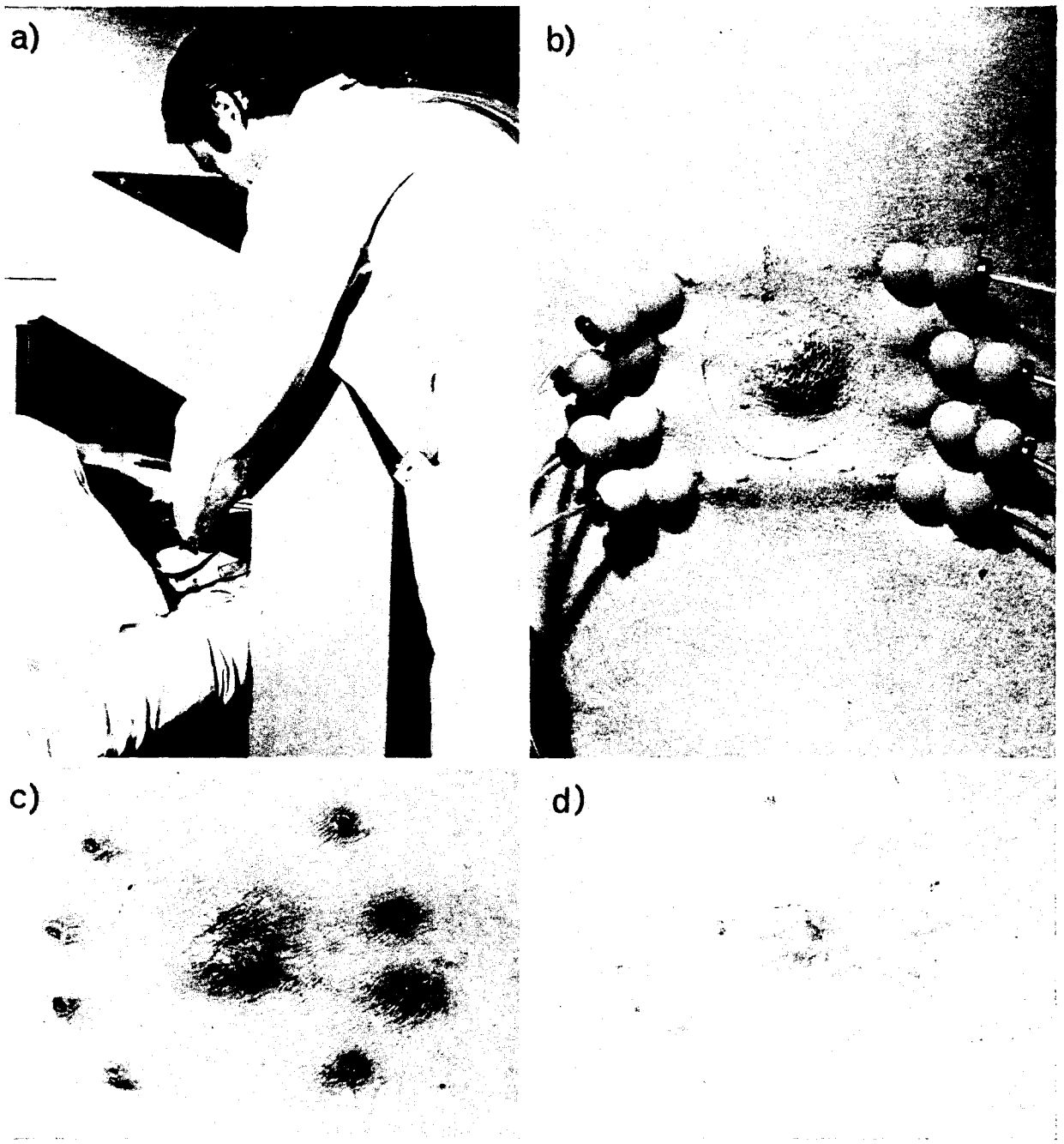


Fig. 1. (a) *Radiotherapist, working behind personnel shield (see text) afterloads californium-252 sources into plastic tubes already implanted into patient in the operating theatre.* (b) *The completed implant; this is patient No.* 4. (*EH*), *the details of the sources used are given in Table* 1. *The sources are held in position in the plastic tubes by solid nylon plugs which are crimped into position by the lead washers which can be seen.* (c) *The appearance of the lesion immediately after removal of the californium sources, approximately five days later.* (d) *The appearance of the lesion three months later; note minimal dry desquamation and virtual disappearance of the lesion.*

(to face p. 366)

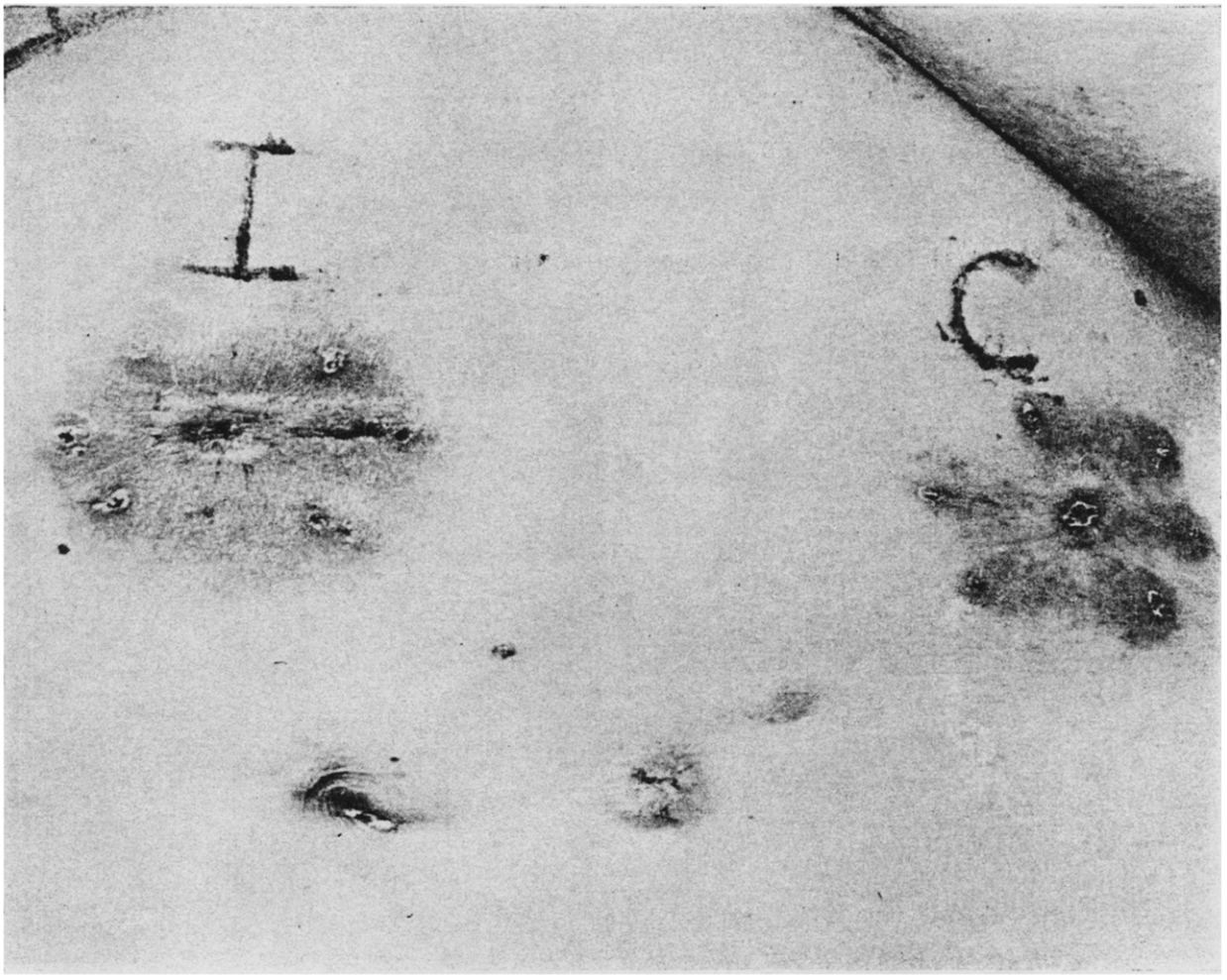


Fig. 2. Peak early skin reaction in patient no. 2 (AC) in whom two comparable lesions on the anterior abdominal wall were implanted with ^{192}Ir and ^{252}Cf, respectively. Note more intense, confluent erythema over the ^{192}Ir treated lesion, but only patchy erythema over ^{252}Cf treated lesion. Later skin reaction over the iridium-treated lesion was also more intense (see text), confirming that the RBE value for the ^{252}Cf neutrons of 7·5, used initially, was too high

Table 1. *Status of patients treated with* ^{252}Cf *implants to* 1 *September*, 1973

Patient	Age	Lesion treated	Previous radiotherapy to area	Implant design	Number of ^{252}Cf sources	Dose to reference point: Planned (effective rad)	Dose to reference point: Neutron dose (rad)	Dose to reference point: Gamma dose (rad)	Total time (hr)	Result
1. MP	56	Recurrent carcinoma of parotid.	Yes, November 1971, 4290 rad/13f/28d ^{60}Co gamma.	3 lines Single plane	7	4250	536	231	93	Transient tumour regression, died with recurrent disease 8 months after implant.
2. AC	88	Superficial anterior abdominal metastasis from ca. breast.	No	3 lines Single plane	7	5400	682	285	103½	Disappearance of lesion, alive with multiple additional skin metastases 10 months after implant.
3. GLC	63	Superficial chest wall metastasis from ca. breast.	Yes, part of implanted area treated July 1970, 4200 rad/10f/22d ^{60}Co gamma.	5 lines Single plane	18	6000	755	325	95	Complete disappearance of lesion, died with cerebral metastasis 8 months after treatment.
4. EH	70	Superficial posterior chest wall metastasis from ca. breast.	No	4 lines Single plane	10	6000	808	341	116½	Complete disappearance of lesion, alive with spinal metastasis 7 months after treatment.
5. HK	59	Chest wall/axillary recurrence from ca. breast.	Yes, April 1969, 4200 rad/10f/45d ^{60}Co gamma.	5 lines Single plane	20	4650	624	290	116	Early moist desquamation, disappearance of treated lesion, alive with recurrence outside treated area 5 months after treatment.
6. KW	77	Carcinoma of breast	Yes, Feb. 1973, 3800 rad/10f/21d 250 kVp X-rays.	7 lines Two plane	26	4700	630	298	89	Badly infected lesion showed minimal early response, alive 3 months.
7. JR	68	Axillary "tail" recurrence from ca. breast.	Yes, Dec. 1969, 3800 rad/10f/45d 250 kVp X-rays.	4 lines Single plane	12	5800	776	337	117	Complete regression of lesion, alive 3 months after implant, but with persistent breast recurrence previously treated by ^{192}Ir implant.
8. FM	71	Axillary node metastasis from ca. breast.	No	7 lines Two planes	16	6000	804	375	127	Early moist desquamation, regression of lesion, 1 month after treatment.
9. IS	69	Recurrent ca. of breast	Yes, Nov. 1968, 3800 rad/10f/21d 250 kVp X-rays.	5 lines Two planes	13	5000	668	296	127	Regression of lesion 1 month after treatment.

than from the ^{192}Ir as is shown in Fig. 2. This early reaction progressed only to dry desquamation and minimal pigmentation over the ^{252}Cf treated area whereas the ^{192}Ir treated area went to moist desquamation and late depigmentation at the central area with a surrounding ring of pigmentation. At two months, the ^{252}Cf treated lesion had shown more marked regression, but by 6 months flattened "ghost" lesions were present in both ^{252}Cf and ^{192}Ir treated areas.

In the light of the findings with the first three patients the lower RBE of 7·0 has been used for the neutron component of the dose for patients Nos. 4–9; the response in patient No. 4 is shown in Fig. 1. In only two patients has the early skin response progressed to moist desquamation, the remainder showed only areas of erythema, and dry desquamation, the remainder showed only areas of erythema, and dry desquamation and transient pigmentation. At this stage it is felt that the RBE value 7·0 approximates fairly closely to the real efficiency of these neutrons in the mixed emission from ^{252}Cf at the dose rates used in clinical implantation of these sources (5–8 rad/hr, neutrons only). At the reference position, the neutron/gamma dose ratio was 2·1–2·4 for all of the implants performed so far, so that because of its lower effectiveness, the contribution of the gamma dose to tissue damage was only 6% of the total effect. Observation of these patients is continuing, with quantitative scoring and photography of skin responses and serial measurement of changes in tumour size. Early tumour regression has been seen in all cases, and the recurrence observed in the implanted area in patient No. 1 (for whom an RBE value of 7·5 was used) may be ascribed to inadequate radiation dose. As yet, no unpleasant surprises have been observed in the response of the normal tissue.

REFERENCES

1. J. T. Brennan, Fast neutrons for radiation therapy. *Radiol. Clin. N. Am.* **7,** 365 (1969).
2. C. H. Paine, Modern after-loading methods for interstitial radiotherapy. *Clin. Radiol.* **23,** 263 (1972).
3. B. Pierquin and A. Dutreix, Towards a new system in curietherapy (endocurietherapy and plesiocurietherapy with non-radioactive preparation). *Brit. J. Radiol.* **40,** 184 (1967).
4. R. D. Colvett, H. H. Rossi and J. Krishnaswamy, Dose-distribution around a californium-252 needle. *Phys. in med. Biol.* **17,** 356 (1972).
5. J. R. Castro, G. D. Oliver, H. R. Withers and P. R. Almond, Experience with californium-252 in clinical radiotherapy. *Amer. J. Roentgenol.* **117,** 182 (1973).
6. R. J. Berry, G. Wiernik and T. J. S. Patterson, Skin tolerance to fractionated X-irradiation in the pig—how good a predictor is the NSD formula? *Brit. J. Radiol.*, **47,** 185 (1974).
7. H. L. Atkins, R. G. Fairchild and J. S. Robertson, Dose-rate effects on RBE of californium and radium reactions of pig skin. *Radiology* **103,** 439 (1972).
8. F. K. Dean, S. C. Bushong, N. Prasad and S. Briney, The relative biological effectiveness of californium-252 on acute mammalian lethality. *Radiology* **104,** 699 (1972).

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Discussion on Preliminary Clinical Experience with Fast Neutron Radiotherapy at Various Centres*

A. SMITH, P. ALMOND and L. DELCLOS (Houston, Texas, U.S.A.): *Evaluation of ^{252}Cf neutron emitter for interstitial and intracavitary radiation therapy*†

SINCE October of 1968, M. D. Anderson Hospital and Tumor Institute under contract with the Atomic Energy Commission has been evaluating ^{252}Cf for use in interstitial and intracavitary radiation therapy [1].

A special storage facility has been designed and constructed of borated water—extended polyester which will accommodate up to 300 μg of ^{252}Cf in the form of afterloading cells, short afterloading cells and applicator tubes. A total of 160 medical sources could be stored at once [2].

OER studies on Chinese hamster ovary cells and RBE studies for acute mortality of mice and for damage to the small intestine of mice have been made [3, 4].

LET measurements have been performed and a computer code—CALRAD—has been written which calculates individual distribution for the neutron and gamma components of the total dose. Complete dosimetric and radiation protection procedures have been established.

Twenty-two patients have been treated with ^{252}Cf implants. Table 1 summarizes the clinical and physics data.

REFERENCES

1. G. D. OLIVER, JR., J. R. CASTRO and P. R. ALMOND, Experience with ^{252}Cf in radiotherapy. *Brit. J. Radiol.* **44,** 527 (1971).
2. J. P. VEERLING, JR., G. D. OLIVER, JR. and E. B. MOORE, A storage and handling facility for ^{252}Cf medical sources. *AAPM Quarterly Bull.* **6,** 92 (1972).
3. F. K. DEAN, S. C. BUSHONG, N. PRASAD and S. BRINEY, The relative biological effectiveness of californium–252 on acute mammalian lethality. *Radiology* **104,** 699 (1972).
4. H. R. WITHERS, G. D. OLIVER and D. W. GLENN, Response of mouse jejunal crypt cells to low dose irradiation of californium neutrons or radium gamma rays. *Radiat. Res.* **48,** 484 (1971).

H. TSUNEMOTO, Y. UMEGAKI, M. URANO, A. KURISU, Y. KUTSUTANI and T. INADA (Anagawa, Japan): *Clinical trials with fast neutrons*

FOR THE clinical applications to be discussed, fast neutrons with a mean energy of about 2 MeV were produced by bombarding a thick beryllium target with 2·8 MeV deuterons. Fast neutron therapy was first tried by Stone, but discontinued because of severe late radiation damage to normal tissues. Re-evaluation of possible advantages is based on recent radiobiological experiments, showing more effective destruction of hypoxic cells in tumours. The aim of our studies was the evaluation of indications and optimum schedules for fast neutron therapy.

Nineteen patients were irradiated between May 1967 and December 1972. Because of the low penetration of the beam, equivalent to 150 kV X-rays, superficial tumours, such as recurrent carcinoma of the breast, metastatic lymph nodes and malignant melanoma, were selected. Of 19 patients, 10 had malignant melanoma, which are frequently classified as the most radioresistant tumours. The treatment results indicate that malignant melanoma can

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*Full addresses of contributors to the discussion can be found in the list of participants to be published in Vol. 10, No. 6, on pp. 393 to 398.

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Table 1. *Californium–252 patients**

Patient	Tumour	^{252}Cf dose† (γ-ray equivalent rad)	Neutron/gamma (rad/hr)	External X-ray treatment	Type implant	Results
1.	Squamous carcinoma, cervix, metastatic to inguinal nodes	6100 (109 hr)	7/3·5		single plane	Local control until death at 8½ months skin-dry desquamation.
2.	Squamous carcinoma, vagina, stage IV	2560 (25 hr)	10/5	5000 R	volume	Distant metastasis at 5 months, local control in pelvis.
3.	Squamous carcinoma, cervix, stage III, metastatic to inguinal nodes.	4000 (100 hr)	5/2·5		single plane	Local recurrence at 10 months.
4.	Squamous carcinoma, lung, metastatic to right submandibular node (4·5 × 3·5 × 2·0 cm)	2260 (40 hr)	7/4		double plane	Died 12 days after implant, of vascular accident —distant metastasis (brain)?
5.	Squamous carcinoma, vagina, stage III.	970 (24 hr)	5/2·8	5000 R	volume	No evidence of disease at 14 months.
6.	Recurrent squamous carcinoma, lower lip (post surgery)	1930 (30 hr)	8/4	5000 R	single plane	Local failure at 2 months
7.	Basal carcinoma, right nasolabial fold.	4250 (67 hr)	8/3·5		single plane	Local recurrence at 5 months.
8.	Squamous carcinoma, lung with skin nodules.	6000 (70·5 hr)	10/10		single plane	Died from distant metastasis at 6 weeks, local lesions much smaller, no moist skin reaction.
9.	Squamous carcinoma, upper lip.	2050 (26 hr)	10/4·5	5000 R	single plane	Local control at 12 months.
10.	Squamous carcinoma, vagina, stage III.	1250 (51 hr)	3/2	5000 R	volume	Local control at 12 months.
11.	Squamous carcinoma, vagina, stage III.	1600 (25 hr)	8/4	5000 R + 1000 R parametrial	volume	Local control at 8 months
12.	Squamous carcinoma, cervix, metastatic to distal urethra.	2790 (69 hr)	5/3	3000 R transvaginal	volume	Died 3 weeks later of cerebral vascular accident.
13.	Recurrent squamous carcinoma (lip) in neck (post X-ray treatment).	4205 (66·7 hr)	8/4		curved plane	Died 9 months later, regression but persistent tumour.
14.	Squamous carcinoma, tongue T_3N_0 (2nd primary)	4000 (70·3 hr)	7/4	2500 R after 3 treatments	double plane	Persistent tumour
15.	Squamous carcinoma, tongue T_3N_2	2700 (67 hr)	5/3	5500 R	volume	Persistent tumour
16.	Metastatic neck node 6 × 6 cm	2220 (44 hr)	6/4·8	6000 R	double plane	Partial regression at 3 months
17.	Squamous carcinoma, skin, metastatic neck node.	2000 (21 hr)	12/8	5000 R	single plane	Persistent tumour at 3 months
		1900 (29 hr)	8/6		single plane	
18.	Melanoma, cervix, vagina	11000 from mucosal dose (2 applications 72 hr each)	5·3/4·0 5·2/5·0		tandem tandem	Regression at 4 months
19.	Melanoma, vagina	7500 (96 hr)	10/8		tandem interstitial + tandem	
20.	Squamous carcinoma, floor of mouth.	2500 (27·3 hr)	13/7	5500 R	single plane	No evidence of disease at 4 months.
21.	Squamous carcinoma, lower lip.	2600 (214 hr)	7/4	5000 R	single plane	Local control at 2 months
22.	Myxoid liposarcoma, right lower thigh.	3000 (214 hr)	2/1	5000 rad† fast neutron (1700 rad $n+\gamma$)	triple plane	Regression at 6 weeks

*Patients listed in chronological order.

†Patients 1–19 "biological" dose was calculated as 7·5 $D_n + 1{\cdot}0\ D\gamma$; patients 20–22 "biological" dose was calculated as 6·5 $D_n + 1{\cdot}0\ D\gamma$

be sufficiently controlled by fast neutron therapy. A malignant melanoma in the right shoulder of one patient, was eradicated by fast neutron therapy, with a schedule of 150 rad per fraction, 2 fractions/3 days. Fast neutron doses were also effective to control a residual malignant melanoma in the right sole, which had been previously treated with 6 MeV X-rays.

In another application of fast neutrons it was shown that adenocarcinoma, such as breast cancer of parotid tumour, and fibrosarcoma were also controlled by fast neutrons within the tolerance limit for normal tissues.

These results indicate that the therapeutic margin could be improved by the application of fast neutrons. Sato *et al.* [1] and Kasuga *et al.* [2] suggested that the radioresistance of B16–C3W melanoma cells might be strongly related to the rapid recovery from sublethal damage in irradiated cells. Fast neutrons which produce relatively less repairable damage might be of value as a modality to control such radioresistant tumours.

In conclusion, it is suggested that tumours consisting of malignant cells which are characterized by intense recovery from sublethal damage or tumours in which reoxygenation is not adequate, might provide suitable objects for fast neutron therapy.

REFERENCES

1. C. Sato, K. Kojima and T. Matsuzawa, *Japan J. Cancer Clinics* **18,** 587 (1972).
2. T. Kasuga, T. Furuse, T. Inada and I. Takahashi, *Japan J. Cancer Clinics* **17,** 380 (1971).

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Achievements to be Expected from New Developments in Tumour Radiotherapy

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Abstract—*A number of possibilities for future improvements of radiotherapeutical tumour treatments are evaluated, with a view of indicating areas where integration of experimental and clinical research might be most fruitful. Such areas are the combinations of radiotherapy with surgery and with chemotherapy, to be based on improved insights into various factors which determine the radioresistance of tumours, e.g. intrinsic cellular radiosensitivity, cell proliferation kinetics, repair of potentially lethal damage, and the presence of anoxic cells. Finally some aspects of time–dose relationships are discussed.*

INTRODUCTION

IT WAS felt appropriate during a symposium on high LET radiations to review the prospects and avenues for research for low LET radiotherapy. The aim of this paper is therefore to discuss a few of the many means which are being investigated for decreasing the proportion of local failures, such as better techniques of irradiation, combination of radiotherapy and surgery, identification of the causes of the tumour radioresistance, alterations in the fractionation and protraction of the radiation treatment to optimize the biological differences between the tumour and the relevant critical normal tissues, and the combination of radiotherapy and chemotherapy.

A. BETTER TECHNIQUES OF IRRADIATION

(*a*) The first step in establishing a radiotherapy protocol is to define the target volume. Its choice involves two factors: (1) delineation of the tumour, (2) identification of the anatomical areas in which there is a significant probability of spread.

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The delineation of the tumour may be improved by many new techniques such as angiography, radioactive isotopes (for instance the use of bone seekers for early detection of bone metastases in breast carcinoma) and the ultra-sound technique. Surgical laparotomy has proved to be useful for the detection of occult abdominal disease in malignant lymphoma and for the study of paraaortic lymph nodes in carcinoma of the cervix.

Careful statistical analysis of the mode of spread of a type of cancer and of the correlations between the characteristics of the tumour (size, histological type, growth rate, etc.) and the probability of occult involvement of the potential contiguous areas of spread may provide an objective basis for the choice of the target volume. For instance, in Hodgkin's disease, it has been shown that for diseases of the upper neck of the lymphoid predominance histological type, the probability of occult abdominal involvement is less than 3%, whereas this probability is higher than 50% for patients with disease in the supra-clavicular fossae of the mixed cellularity histological type. In breast cancer the probability of involvement of the axillary lymph node is practically zero when the biopsy of the lower external mammary chain is negative.

(*b*) Once the target volume has been defined, the next step is to find the optimal technique of irradiation. The pattern of dose distribution has a great clinical importance, as the tolerance of a normal tissue is influenced both by the dose and by the size of the irradiated volume. The dose to the tumour mass should be uniformly high, without zones of underdosage. In some cases it may be useful to give a higher dose to the central part of the tumour.

The protocols of irradiation which are presently used, are often crude and the pattern of dose distribution might, in many cases, be considerably improved by a sophistication of the technique, such as individualized beam shaping by especially tailored lead blocks, combination of various types of radiation (for instance photons and electrons), or association of beam therapy and interstitial therapy, a combination that has been made more reliable through the help of computer dosimetry.

Computer dosimetry will probably make possible the study of dose distribution in 3 dimensions, a significant advance because calculation of only a section through the centre of the tumour may leave undetected significant inhomogeneities. Furthermore, non-coplanar beams may have, for certain tumour localizations, important advantages. Their use is presently prevented by dosimetric problems, which can be solved by computer dosimetry.

In the beam therapy the use of a large number of fields (of various sizes, shapes, weights, etc.) might often considerably improve the dose distribution, but is prevented by the fear of an increased risk of errors during the set up of the patient. Automation of the set up of the patient during irradiation would facilitate the use of complex irradiation techniques and deserves further research even if automated generators might be expensive. It must be associated with more precise positioning and immobilization of the patient. Constant check of this position by automatic devices should also be considered. Finally, computer optimization of the dose distribution remains an important goal for long term research.

B. COMBINATION OF RADIOTHERAPY AND SURGERY

Clinical experience has shown that two main factors are correlated with the dose required for tumour control: (i) The histological type of the tumour, (ii) The size of the tumour.

It is well known that for most types of tumours, the dose required for tumour control increases with the size of the tumour. For instance, Fletcher [1] reported that the dose that controls 90 per cent of the squamous cell carcinomas of the supraglottic, larynx, and tonsillar fossa is 6000 rad in 6 weeks for tumours of less than 2 cm, 7300 rad in 7 weeks for tumours of 4 to 6 cm, and 7890 rad in 8 weeks for tumours larger than 6 cm. On the other hand, 5000 rad in 5 weeks is sufficient for subclinical disease in the lympatics of the neck.

When a large dose is required, which would not be tolerated by surrounding normal tissues, the following procedures could be used: (i) progressive reduction of field size during treatment as advocated by Baclesse; (ii) boosts to residual tumour; and (iii) combination of radiation with a conservative surgery procedure for the removal of gross masses and post-operative irradiation of remaining subclinical aggregates of tumour cells.

The advantages of this last technique have been recently stressed by Fletcher [1]. The surgeons can remove a tumour even when very big, but unless he performs a superradical procedure that is very mutilating and not always effective, he often leaves microscopic disease near the line of resection. On the other hand, radiation eradicates small cancer foci with relatively limited doses (5000 rad in 5 weeks) that are tolerated even when delivered on large target volumes. Among the cancers which would benefit from such combination treatments, Fletcher listed locally advanced breast cancer, advanced cancer of the cervix, advanced cancer of the ovary, and some types of cancer of the head and neck. Cancer of the bladder could be added to the list. Furthermore, tumourectomy and irradiation appears for small breast cancer an effective treatment that gives good cosmetic results.

In order to avoid unnecessary irradiation, some authorities have proposed waiting until the subclinical disease has become apparent before irradiating. However, gross recurrences are more difficult to control than subclinical disease, as shown for instance by data of Fletcher and Evers [2] on head and neck cancer. As local recurrences are frequent after conservative surgery, systematic prophylactic irradiation is therefore very beneficial.

C. IDENTIFICATION OF THE FACTORS OF RADIORESISTANCE

1. *Cell radiosensitivity*

It is not yet known whether clinical radioresistance could be due to a low radiosensitivity of tumour cells. For instance the cell survival curve of one human melanoma measured *in*

vitro [3] has parameters ($N = 2$ and $D_0 = 180$ rad) which are similar to those of other human tumour cells. However, only a few human tumour cells have yet been studied; moreover the relevance to radiotheray of conventional *in vitro* cell survival curves has been questioned.

The cell survival is measured on isolated single cells under conditions where there are no cellular interactions and no vascular factors. The distribution of the cells in the phases of the cell cycle differ *in vivo* and *in vitro*. Finally the initial part of the curve is not accurately known, although it is the most important for fractionated radiotherapy.

In fact, many data [4, 5] suggest that for human cells *in vivo* the cell survival curve has an initial slope which might correspond to the non-reparable damage, single-hit, component. A difference in the initial slope can explain wide variations in the response to fractionated radiotherapy. Let us consider for instance two populations, A and B, of cells with a same extrapolation number ($N = 5$) and ($D_0 = 150$ rad). If the population A has an initial slope corresponding to a $D_1 = 300$ rad and the B population has no initial slope (multi-target single-hit model), the surviving fraction would be similar in the two populations after 5 sessions of 500 rad ($5 \cdot 10^{-5}$ for A and $10 \cdot 10^{-5}$ for B). If the session doses are small, the effects differ greatly. After 14 sessions of 200 rad, the surviving fraction would be $6 \cdot 10^{-5}$ for population A, and 0·24 for population B.

This example demonstrates how important it would be to have data on the initial slopes of at least the critical normal tissues in order to choose the optimal dose per session. The initial slope of the survival curve measured for an asynchronous cell population is that of the cells which are in the most sensitive phase of the cell cycle and is therefore influenced by the kinetic state of the cell population.

2. *Cell population kinetics*

Actually it seems likely that differences in radiosensitivity among tumours are at least partly due to differences in the kinetics of cell proliferation during undisturbed growth and during radiotherapy and to differences in tumour vascularization.

In order to compare the various histological types of tumours, we have collected from the literature and from our own patients data on the growth rate of untreated lung metastases [6]. Despite a wide range of doubling times within each histological type of tumour, the mean values differ significantly (Table 1). Subsequently, we also collected data on the labelling index (L.I.) of these 5 histological types of tumour [7]. These also differ significantly although there is a wide range of values in each group. There is a strong correlation between the L.I. and the doubling time [7]. This has been confirmed recently on a group of breast cancers in which L.I. and the doubling times were measured in the same patients. The L.I. can be used for the evaluation of the proliferative fraction (assuming $T_{cS}/T_c = 0{\cdot}33$).

Table 1. *Mean kinetic parameters of various histological types of human tumours**

Histological type	Doubling time (days)	Labelling index (%)	Growth fraction (1) (%)	Cell loss factor (2) (%)	Absolute cell loss per 10^9 cel. per day (3)	Cell Renewal rate per cent per day (4)	Radiosensitivity (Mean tumour control dose in rad)	Chemosensitivity
Embryonal Tumours	27 (76)	30 (6)	90	93	420–10^6	42	2500–3000	+ +
Malignant Lymphoma	29 (41)	29 (15)	90	94	510–10^6	51	3500–4000	+ +
Mesenchymal Sarcoma	41 (87)	15 (68)	15	76	55–10^6	5·5	8500	—
Squamous cell Carcinoma	58 (51)	8 (68)	25	89	100–10^6	10	6000–7000	+
Adenocarcinoma	83 (134)	2 (121)	6	56	11–10^6	1	6000–8000	±

* (See 6, 7)

The numbers in brackets indicate the number of patients for whom data were available

(1) Calculated from L.I, assuming $T_c/T_S = 3$.

(2) Calculation based on doubling time and labelling index, as defined by Steel [8] assuming $T^S = 15$ hr and $\lambda = 0{\cdot}7$

(3) Equal to the product: birth rate × cell loss factor.

(4) Equal to: absolute cell loss/number of cells.

Assuming for all tumour cells a duration of 15 hr for the *S* phase, and knowing the labelling index, one can calculate the potential doubling time. Comparison of the potential and the actual doubling time provides the cell loss factor [8]. Cell loss can also be expressed as the per cent cell loss per day, which is nearly equal to the cell turnover rate per day.

Table 1 shows that there is a significant correlation between on the one hand the proliferative fraction and/or the cell loss and, on the other hand, the chemosensitivity or the tumour control dose. The only tumours that yet have been controlled by drugs belong to the two histological types with a high proliferative fraction and a large amount of cell loss (malignant lymphoma and embryonal tumours). This correlation between proliferative fraction and chemosensitivity might be explained by the fact that the *S* phase is, for most drugs, the most chemosensitive phase. Furthermore cell loss appears to be a factor in drug sensitivity [9].

The correlation between cell kinetics and radiosensitivity that is shown in Table 1 is more difficult to explain. It could be argued that this correlation is fortuitous. This is unlikely. Moreover, Breur [10] evaluated for 16 patients with lung metastases of various histological types an index of radiosensitivity after fractionated radiotherapy, based on the reduction of the volume of the metastases on successive chest roentgenograms. He demonstrated a strong correlation between the growth rate and the radiosensitivity whatever the histological types of the tumour. In 2 series of patients with lung metastases irradiated with single doses [11, 12] a similar correlation was found. Furthermore, Hermens [13] recently reviewed the data available for experimental tumours and concluded that of all the tumour characteristics considered, the proliferative fraction is the one that is best correlated with specific growth delay. The latter parameter is itself linked with radiosensitivity.

The correlation between the parameters of growth and radiosensitivity that is observed in all these data, confirms the old, so called, law of Bergonie and Tribondeau [14] which stated that the most radiosensitive tissues are those with a high mitotic index.

If this correlation seems established, its interpretation remains difficult. Several factors must be considered:

(*a*) *Quiescent cells.* The proportion of quiescent cells or of cells with long cell cycle times is higher in slowly growing tumours. It is not known whether this could be a factor of radioresistance. The data about the radiosensitivity of quiescent cells are scarce and conflicting [see 15]. In some normal tissues, for instance the liver, quiescent cells appears to be less radiosensitive than actively proliferating cells. *In vitro* the radiosensitivity of quiescent cells varies with the strain of cell and the factors which cause the cell quiescence [16]. In spheroid tissue culture, the cells in G_1 phase are relatively sensitive and this is the phase in which most of the quiescent cells are [17].

(*b*) *Repair.* Repair of potentially lethal damage (p.l.d.) might contribute to explain the relative radioresistance of slowly growing tumours. It is well known that *in vitro* and *in vivo*, the cells remaining quiescent during several hours after irradiation have a higher survival. P.l.d. is repaired mainly during G_1 phase and lesions are expressed if cells enter *S* phase before this damage is repaired. Recent data [18] have demonstrated that small rapidly growing NCTC 2472 tumours show slight p.l.d. repair capacity, whereas an important p.l.d. repair similar to that found in stationary phase culture, is observed when the tumours are large and slowly growing. Zinninger and Little [19] have analysed, for cells in exponentially growing tissue culture or in stationary phase, the influences of fraction size and of elapsed time between fractions. Stationary cultures represent an experimental model model for large tumours and exponential cultures simulate regenerating normal tissue. When the doses per fraction are high (1000 or 500 rad) or the elapsed time short (1 or 2 days) the surviving fraction is smaller for exponentially growing cells. The reverse is true when the fraction size is small (333 rad or 200 rad) or the elapsed time longer (3 or 4 days). This is probably due to 2 factors: (i) The amount of p.l.d. repair found after low single doses (200–300 rad) is less than for higher doses (500–1000 rad); and (ii) Repopulation is more important in exponential cultures and when the time interval between fractions is longer.

(*c*) *Repopulation.* Repopulation has been demonstrated in a few experimental tumours [13, 15, 20, 21] and might be a cause of radioresistance during a fractionated course of radiotherapy [20]. Throughout such a treatment the repopulation rate varies both in experimental tumours [20] and in normal tissues, such as skin [22]. Repopulation becomes important in both tissues after the second week and it is more rapid in skin. This contributes to the relative sparing of the skin.

A few exceptional human tumours grow despite iterative irradiation. This is the case for Burkitt's tumour when the time interval between fractions is too long [23]. Repopulation has been shown in lung metastases after a single irradiation [11, 12]; however, there is no evidence of a higher repopulation rate in radioresistant tumours. Preliminary unpublished data on the time course of changes of the labelling index (L.I.) in human tumours after irradiation suggest a wide range of responses in tumours of a same histological type. Moreover, in order to be a factor of radioresistance for slowly growing tumours, the repopulation rate would have be higher in those tumours than in rapidly growing tumours; this is not impossible but it is unlikely.

(*d*) *Percentage of anoxic cells and reoxygenation.* Thomlinson [24] found a higher proportion of anoxic cells in rapidly growing experimental tumours but there are not data for human tumours. Reoxygenation seems correlated with at least two factors, the decrease in oxygen consumption by irradiated cells and the shrinkage of the tumours. The removal of dead cells is slow in tumours with a low cell turnover and Denekamp [25] showed that post-irradiation shrinkage is more rapid in tumours documented as having a high cell loss factor prior to irradiation. In contrary to what is observed in experimental tumours, there is in human tumours a positive correlation between growth fraction and cell loss [7]. Reoxygenation may therefore be more rapid in tumours with a high proliferative fraction.

(*e*) *Host immunological defenses.* For many experimental tumours, the tumour control dose (t.c.d.) is much smaller than the dose which would leave one surviving cell in the tumour [26]. T.c.d. is increased when the host immunological defenses are reduced by total-body irradiation or chemotherapy. The correlation between immunological defenses and tumour growth rate is complex. Growing tumours are in competition with the immunological responses but the tumours seem to act as a depressor by diminishing the effectiveness of the cell mediated response and by eliciting the production of substances which block antibodies (antigen overexposure). After surgical removal [27] or local irradiation the resistance reappears and may reduce the dose required to achieve the destruction of the tumour. The growth rate or the shrinkage rate may then be somewhat correlated with the t.c.d. Unfortunately, there are, at present, no data for human tumours.

In summary, repair of potentially lethal damage and reoxygenation are the factors which are the most likely to explain the relative radioresistance of slowly growing tumours. It should be emphasized on the other hand that local control and cure cannot be equated, as rapidly growing tumours may have greater propensities to metastasize.

D. TIME–DOSE RELATIONSHIP

The multiplicity of the factors involved in time–dose relationship explains the difficulties in the choice of the optimal fractionation. The data of Du Sault [28] on the control of mammary tumours of C3H mice with different schemes of irradiation, illustrate the complexity of the problem. For a dose per fraction of 400 rad, the proportion of cures decreased progressively when the time interval was increased, which showed the influence of repopulation. For a dose per session of 1000 rad, the best results were obtained with an interval of 2·5 days. This probably is a result of the combined influences of reoxygenation and repopulation. Comparison of low LET and high LET fractionated radiotherapy on comparable groups of experimental and human tumours might help to analyse the respective influences of these two factors.

The scheme of fractionation must take into account all these factors not only for the tumour, but also for the relevant critical normal tissues. For instance, long time intervals between sessions are of interest when reoxygenation of tumour cells is a slow process, and/or when the rate of repopulation is higher in the critical normal tissue than in the tumour, but it should not be forgotten that the rate of repopulation varies under fractionated irradiation. Small doses per session are of interest when (i) the slope of the initial part of the survival curve is greater for the tumour cells than for the cells of the critical normal tissue; (ii) there are many quiescent cells in the tumour (because repair of p.l.d. is less important for small doses); and (iii) the proportion of anoxic cells is high. In fact, the problem is to recognize to which category a patient belongs and to select for him the optimal fractionation. Empirical formulae [29] are useful for comparing data within a limited range of fractionation, but do not help to understand, predict, or optimize. Mathematical models have been built in order to incorporate large amounts of clinical data and to enable their use. However, in order to be workable they must oversimplify the biological problems. For instance Cohen's model [5] has seven

parameters. This relatively large number results in the fact that many sets of values would fit the clinical data. But despite this multiplicity of parameters the model has to neglect such parameters as oxygen factors, variation in rate of regeneration during treatment, etc. Furthermore, all the formular and models must use the available clinical data, which are imprecise and few. These come mainly from retrospective studies hampered by many biases. The tumours are heterogeneous with regard to size, location, growth rate, and, sometimes, histological type. The data from various observers have to be pooled and the methods of analysis often have major pitfalls. Controlled prospective studies on homogeneous groups of patients could give valid answers, but they are time consuming and not always rewarding.

This discussion stresses the need for further studies and for close integration of clinical investigation and experimental approach, and underlines the relevance of cell kinetics from this point of view. As tumour cell kinetics and histological types are interrelated, the latter may be of guidance, but remains insufficient since there is a wide range of growth rates within each histological type. More direct approaches such as *in vitro* studies of tumour specimens are needed but still unavailable. Their search is one of the important goals of radiotherapeutic research. Meanwhile, fractionation should be based on the tolerance of the relevant normal critical tissues which is similar for all patients, rather than on the characteristics of tumours which are difficult to evaluate.

Low dose rate irradiation is another field for research. Clinical data on external beam therapy have shown that its effectiveness is not due to the pattern of dose distribution achieved with interstitial or brachytherapy but rather to radiobiological factors. [30]. The differential effect between tumours and normal tissues appear to be enhanced by continuous low dose rate irradiation, as the cell survival in experimental tumours is lower than expected, which is probably due to cell reassortment [31], and to reoxygenation [32]. However, there seems to be some discrepancy between early and late effects on normal tissues and further work is needed to study the late effects on connective tissue and vascularization.

E. RADIOSENSITIZERS AND COMBINATIONS OF RADIOTHERAPY AND CHEMOTHERAPY

Radioprotectors and radiosensitizers have no practical interest when the same degree of effect is achieved on tumour and normal tissues. On the other hand, preferential protection of well vascularized tissues or preferential sensitization of hypoxic cells would be of major clinical interest. The latter has been recently achieved *in vitro* and *in vivo* [33].

Combination of radiotherapy and chemotherapy has two main purposes: (i) increase in the frequency of tumour control, and (ii) sterilization of sub-clinical disease outside the target volume. Some clinical data suggest the value of such combination, notably for Hodgkin's disease [34] or Wilm's tumour. Actually these two treatments are complementary: (i) the cells in *S* phase are radioresistant whereas they are sensitive to proliferation-specific drugs. Quiescent cells are insensitive to most drugs but can be killed by radiation. Drugs are able to reach cells in the undervascularlised zones of the tumour. (ii) The critical tissues for the two agents are different: normal tisses with a high cell turnover rate for drugs, connective tissue in the vicinity of the tumour for ionizing radiations.

Unfortunately combination therapy sometimes enhances the effect not only on tumour cells but also on normal tissues. The differential effect of combination therapy could be improved either by decreasing the effect on normal tissues or by increasing the effect on tumours. Two avenues of research are being explored:

(a) Cell reassortment or semi-synchronization

After a selective killing with a phase dependent drug such as cytosine arabinoside which kills the cell in *S* phase, the surviving cells, are distributed in the other phases of the cell cycle. When the surviving cells progress throughout the cell cycle the reassortment will result in a cyclic variation of chemo- or radiosensitivity.

This effect may be more pronounced if the drug provokes also a temporary block of the cell cycle. For instance, hydroxyurea has a selective effect on cells in *S* phase and blocks the cell at the boundary between G_1 and *S* phase. The semi-synchronisation may be further enhanced when the depletion due to cell killing causes a recruitment into the cycle of quiescent cells.

However, these phenomena occur both in tumours and in normal tissues such as bone marrow [35] or intestine. Reassortment would be of interest from a therapeutic point of view only if the timing of synchronization were different for the critical normal tissues and the tumours. This may occur but the timings are

often similar. The choice of the proper time interval is therefore very critical and the amount of information required to make such a procedure feasible is substantial. Meanwhile the time interval between drug administration and irradiation should be based on the kinetic data of normal tissues.

(b) Repopulation

Cycle dependent drugs have a more selective effect on tumour cells initially during the first few hours after irradiation or drug administration, that is before the recruitment of normal bone marrow stem cells. It is again more selective later, after the completion of regeneration in the critical normal tissues such as the bone marrow and the intestine, as the duration of the regeneration of normal tissues seems, in mice, to be shorter than the phase of accelerated repopulation which occurs in the tumour after treatments.

Data for human tumours are lacking, but the analysis of the time course of the variation of the volume of human metastases after irradiation suggest that accelerated growth may last for about one month [12]. This would offer an opportunity for combination therapy with a cycle dependent drug. Further work is needed along these lines before these concepts could be applied to human therapy.

In conclusion, a better distribution of dose in space and in time may enhance the differential effect on neoplastic tissues. Experimental studies suggest ways of improving local control of tumours, but extrapolation from experimental tumours and, in particular transplanted tumours, to human tumours is difficult and a close integration of experimental and clinical research is a prerequisite to any further progress.

But these efforts should not obscure the fact that even in the more developed countries the standards of radiotherapy practice are not equally high in all therapeutic centres. Education is the only way to bridge the gap between the more advanced centres and the others, and might be, during the next decade, the most efficient way of improving the overall radiotherapeutic results.

Furthermore it should not be forgotten that between 2/3 and 3/4 of the failures are due to dissemination of disease outside of the target volume. Prophylactic irradiation of the areas where the presence of subclinical occult disease is the most probable, and treatment combining irradiation with chemotherapy and/or immunotherapy offers, from this point of view, important potentialities for improving cure rates, which deserve further investigation.

REFERENCES

1. G. H. Fletcher, Radiation therapy and subclinical disease. (In press), (1973).
2. G. H. Fletcher and W. T. Evers, Radiotherapeutic management of surgical recurrences and post-operative residuals in tumours of the head and neck. *Radiology* **95,** 185 (1970).
3. J. Weininger, C. Aubert and E. Malaise, (Unpublished results).
4. J. Dutreix, A. Wambersie and C. Bounik, Cellular recovery in human skin reactions. Application to dose fraction numbers overall time relationship in radiotherapy. *Europ. J. Cancer* **9,** 159 (1973).
5. L. Cohen, Cell population kinetics in radiation therapy. Optimization of tumor dosage. *Cancer* **32,** 236 (1973).
6. A. Charbit, E. Malaise and M. Tubiana, Relation between the pathological nature and the growth-rate of human tumours. *Europ. J. Cancer* **7,** 307 (1971).
7. E. Malaise, N. Chavaudra and M. Tubiana, The relationship between growth rate, labelling index and histological type of tumours. *Europ. J. Cancer* **9,** 305 (1973).
8. G. G. Steel, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer* **3,** 381 (1968).
9. K. D. Bagshawe, Tumour growth and antimitotic action. The role of spontaneous cell losses. *Brit. J. Cancer* **22,** 698 (1968).
10. K. Breur, Radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 173 (1966).
11. H. A. van Peperzeel, Effects of single doses of radiation on lung metastases in man and experimental animals. *Europ. J. Cancer* **8,** 665 (1972).
12. E. Malaise, A. Charbit, N. Chavaudra, P. F. Combes, J. Douchez and M. Tubiana, Changes in volume of irradiated human metastases. *Brit. J. Cancer* **26,** 43 (1972).

13. A. F. Hermens, Variations in the cell kinetics and the growth rate in an experimental tumour during natural growth and after irradiation. Thesis, Amsterdam (1973).
14. J. Bergonie and L. Tribondeau, Interpretation de quelques resultats de la radiotherapie et essai de fixation d'une technique rationelle. *C.r. Acad. Sci* **143,** 983 (1906)
15. M. Tubiana, The kinetics of tumour cell proliferation and radiotherapy. *Brit. J. Radiol.* **44,** 325 (1971).
16. G. M. Hahn and J. B. Little, Plateau phase culture of mammalian cells. An *in vitro* model for human cancer. *Current Top. Radiat. Res. Quart.* **8,** 39 (1972).
17. C. E. Durand and R. M. Sutherland, Dependence of the radiation response of an *in vitro* tumor model on cell cycle effects. *Cancer Res.* **33,** 213 (1973).
18. J. B. Little, G. M. Hahn, E. Frindel and M. Tubiana, Repair of potentially lethal radiation damage *in vitro* and *in vivo*. *Radiology* **106,** 689 (1973).
19. G. F. Zinninger and J. B. Little, Fractionated radiation response of human cells in stationary and exponential phases. *Radiology* **108,** 423 (1973).
20. 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 (1970).
21. M. Tubiana, E. Malaise and E. Frindel, Repopulation et facteur temps en radiotherapie. *J. Radiol Electrol.* **52,** 847 (1971).
22. J. Denekamp, Changes in the rate of repopulation during multi-fraction irradiation of mouse skin. *Brit. J. Radiol.* **46,** 381 (1973).
23. T. Norin, P. Clifford, J. Einhorn, N. Einhorn, B. Johansson, G. Klein, J. Onyango, A. de Schryver and R. Wolstam, Conventional and super-fractionated radiation therapy in Burkitt's lymphoma. *Acta Radiol.* **10,** 545 (1971).
24. R. H. Thomlinson, The oxygen effect and radiotherapy with fast neutrons. *Europ. J. Cancer* **7,** 139 (1971).
25. J. Denekamp, The relationship between the cell loss factor and the immediate response to radiation in animal tumours. *Europ. J. Cancer* **8,** 335 (1972).
26. S. Rockwell and R. F. Kallman, Cellular radiosensitivity and tumor radiation response in EMT6 tumor cell system. *Radiat. Res.* **53,** 281 (1973).
27. G. Barski and J. K. Youn, Evolution of cell mediated immunity in mice bearing an antigenic tumour. Influence of tumour growth and surgical removal *J. nat. Cancer Inst.* **43,** 111 (1968).
28. L. du Sault and P. P. Maximuke, Limitation on calculation of dose based on number of fractions. *Radiology* **105,** 191 (1972).
29. F. Ellis, Dose-time and fractionation: a clinical hypothesis. *Clin Radiol.* **20,** 1 (1969).
30. B. Pierquin and F. Baillet, La teleradiotherapie continue et de faible debit. *Annales de Radiologie* **14,** 617 (1971).
31. E. Frindel, G. M. Hahn, D. Robaglia and M. Tubiana, Responses of bone marrow and tumor cells to acute and protracted irradiation. *Cancer Res.* **32,** 2096 (1972).
32. H. B. Kal and G. W. Barendsen, Effects of continuous irradiation at low dose rates on a rat rhabdomyosarcoma. *Brit. J. Radiol.* **34,** 252 (1972).
33. J. Denekamp and B. D. Michael, Preferential sensitization of hypoxic cells to radiation *in vitro*. *Nature, New Biol.* **239,** 21 (1972).
34. M. Tubiana and G. Mathe, Combined radiotherapy and chemotherapy in the treatment of Hodgkin's disease. *Series Haematologica* **6,** 205 (1973).
35. F. Vassort, M. Winterholer, E. Frindel and M. Tubiana, Kinetic parameters of bone marrow stem cells using *in vivo* suicide by tritiated thymidine or by hydroxyurea. *Blood* **41,** 789 (1973.

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Registration of Clinical Data and Criteria for Selection of Patients for Fast Neutron Trials

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Abstract—*The difficulties of conducting satisfactory clinical trials of fast neutron therapy are outlined. There is a need to establish an organisation for collaborative multi-centre trials to provide evidence of objective evaluation of fast neutron therapy in a reasonable period of time. A minimal list of variates is suggested for record purposes in such collaborative trials. It is also advised that a registry of late radiation morbidity be maintained to ensure early recognition of any associated untoward effects of fast neutron radiation.*

IT HAS BEEN shown by the presentations today that fast neutrons may be used in the management of cancer patients with reasonable safety and produce impressive regression of large tumours [1].

It yet remains to be seen whether fast neutrons offer any real improvement compared to the use of photons, electrons or protons in cancer therapy. The yardstick is the measure of local control of tumour in the treated area compared with the level of normal tissue reaction produced, particularly late high-dose effects or normal tissue necrosis. Can neutrons increase the therapeutic ratio?

The difficulties of conducting random clinical trials of fast neutron therapy were well discussed at the first meeting at Rijswijk [2]. The problems fall into four categories; ethical, biological, physical and statistical.

The ethical considerations demand a value judgement to be made of the benefit the patient may obtain from the new radiation compared to a conventional radiotherapy programme. At the present time only patients who have a low probability of cure are considered suitable for such trials of fast neutrons and they are less than ideal clinical material. The biological criteria of importance include an influence that foci of anoxic cells may be present in the tumour and that anoxia may determine a poor response to X-rays. The tumour has to be locally advanced and yet have a low probability of having shed distant metases. A reasonably long expectation of life is required to allow for an adequate period for assessment of radiation effects after treatment.

The physical factors concern both the tumour and the treatment facility. Firstly the tumour should be accessible to allow its response to be measured. The location of the tumour must be such that adequate depth–dose and dose-rates may be obtained from the neutron facility and an arrangement of treatment fields possible to provide good distribution of dose around the cancer.

Finally the statistical problem is simply one of obtaining sufficient numbers of patients in such a clinical trial to be able to come to a significant conclusion about the merits of the new treatment in a period of, say, 5–7 years.

These problems are not readily overcome and it would seem that solutions may have to be found in co-operative multi-centre trials. I should say, at once, that where it is possible for

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a single radiotherapy unit to undertake definitive clinical trials independently, then they should be given every encouragement to do so because of the obvious advantage of interval consistency in their management and assessment. However this is not possible in the evaluation of three specific areas of the investigation into the use of fast neutrons.

Firstly the evaluation of the effects of fast neutrons on uncommon tumours which respond poorly to low–LET radiations (Table 1), requires collaborative trials.

Table 1. *Evaluation of fast neutron therapy*

Ucommon tumours suitable for co-operative trials
Malignant melanoma
Metastatic teratoma testis
Osteogenic sarcoma
Soft tissue sarcoma
Post-cricoid carcinoma
Salivary gland carcinoma

Secondly if preliminary clinical investigations show that real improvement is obtained in patients with locally advanced tumours, studies will later have to be made of the effects of fast neutrons compared to X-rays on early cancers which have a high probability of cure by X-rays. It is obviously important in this situation to have such large numbers of patients that an answer be obtained as soon as possible and this can only be realised by collaborative clinical trials.

As Dr. Catterall has shown [1] to illustrate a significant difference of 15% in local control rate, from 10 to 25%, less than 50 patients are required in the treated and control groups. When we come to put neutron therapy to the test in the management of early cancer of the larynx in which an 80% cure rate is presently expected using X-rays, demonstration of an improvement at this level of only 10% requires over 200 patients in both the experimental and conventionally treated groups.

I have mentioned two reasons for requiring collaborative clinical trials—firstly in order to gain experience in the treatment of rare tumours—and secondly, in order quickly to conduct definitive trials in patients with common tumours which have a high probability of cure.

There is a third and no less important reason for establishing a co-operative organisation; that is to provide a registry of radiation morbidity. This is especially important when individual centres will have small number of long terms survivors following initial clinical trials. It is only by collecting and collating the data from world-wide experience that one can hope to learn of any important intermediate or late complications.

When I was given this topic, the organisers asked me to consider five topics:

1. Information about patients
21 Information about tumours
3. Details of treatment schedules
4. Evaluation of tumour effects
5. Measurement of normal tissue responses

I feel that the data collected in any initial co-operative study should be limited and I have prepared what I regard as a minimal list of variates, so that the clerical burden of collaborators is reduced to the registration of essential data.

First of all there is the basic administrative data of the patient's name, age and sex with a record of the collaborating hospital and radiotherapist. Table 2 lists the information required about the tumour; the site of origin, the histological diagnosis and a record of the international TNM staging. The treatment state indicates if the tumour is previously untreated, or post-operative, had previous X-ray therapy or perhaps cancer chemotherapy. It is important that there be a brief statement of tumour site to be treated, whether it be primary, lymph nodes or other metastatic lesion.

Table 2. *Evaluation of fast neutron therapy*

Clinical data		
Organ/Tissue		
	Histological diagnosis	
	Stage T N M	
	Treatment state	
	Primary	Site and size
	Nodes	Site and size
	Metastases	Site and size

Table 3 concerns treatment data required for assessment of results. Details of the site treated, with a note on whether the treatment includes the primary alone, primary together with nodes, lymph nodes alone or another metastatic site.

Then is recorded the treatment technique, field sizes used and a calculation of the high dose volume. We also require a record of the treatment facility and scheme of fractionation stating the number of fractions, the interval of time between fractions and the overall time.

Table 3. Evaluation of fast neutron therapy

Treament data		
Site	T : T+N : N : M	
Treatment facility		
Field size	Technique	
High dose volume		Date of 1st treatment
Number of fractions	Interval	Overall time
Tumour dose	Central	Maximum Minimum
Normal tissue dose		Maximum
Critical organ dose		Maximum

The tumour dose is then recorded together with the maximal normal tissue dose and the dose received by a critical organ.

A record of the follow-up data must include the patients survival or date of death and a note of the state of the treated tumour and other related disease. Finally an assessment of radiation morbidity—early and late reactions—should be recorded.

At the present time most clinical trials record the tumour response in an "all or none" way indicating complete regression or the presence of residual disease. When so many advanced tumours are being treated in these trials I feel much valuable information may be lost if we continue to record results in this way.

I think it would be rewarding to devise and use an arbitrary scale to record tumour response. The assessment would not be wholly subjective as accurate measurement of tumour dimensions either directly or radiographically may be possible in most patients.

In certain departments more detailed quantitative studies may be made following the example work on the response of lung metastases by treating subcutaneous metastases of bowel and breast cancers.

Finally we have to consider the measurement of normal tissue reactions where it seems perfectly valid to use arbitratry scales for skin and mucosal reactions, which have already been used in many centres. It cannot be too highly stressed that the detailed assessment of normal tissue reactions are as important as assessment of tumour response in evaluating a new form of treatment. Normal tissue reactions must be equated in both groups of patients in the trial. Only then can a *real* improvement in tumour control be established.

Much discussion has centred on the topic of the high RBE values which have been found with low doses of fast neutrons. It is therefore important that the evaluation of fast neutron therapy includes a registry of intermediate and late effects (Table 4). This would provide, if all centres using neutrons clinically would co-operate, an early warning system to ensure that any untoward effect may be detected quickly and its relationship to this new form of treatment either confirmed or refuted. Without such co-operation it will be impossible for individual centres to determine any significant "cause and effect" relationship for a long time indeed.

Table 4. Evaluation of fast neutron therapy

Late radiation effects
Skin and soft tissue damage
Lens opacification
Hypoplastic anaemia
Leukaemia
Neoplasia
Gonadal response

It is still unknown if fast neutrons do increase the therapeutic ratio in the management of cancer and if this is shown its indications will have to be carefully defined. This is only likely to result if close collaboration develops between centres interested in this experimental work. In addition the careful collection of data will certainly also be rewarding in improving our understanding of the action of X-rays and perhaps also result in their more rational application, as well as being essential for the objective evaluation of fast neutron therapy.

REFERENCES

1. M. CATTERALL, The treatment of advanced cancer by fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital, London. *Europ. J. Cancer* **10,** 343. (1974).
2. Proceedings of the 1st Meeting on Fundamental and Practical Aspect of the Application of Fast Neutrons in Clinical Radiotherapy. *Europ. J. Cancer* **7,** 97–267 (1971).

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International Cooperation with Regard to Clinical Trials of Fast Neutron Radiotherapy

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Abstract—*A review is presented of various problems which require international cooperation between radiotherapy centres in order to derive insight into the value of fast neutron radiotherapy within the shortest time and with the smallest expenditure of money and effort possible.*

A CONSIDERABLE amount of quantitative data is now available from radiobiological experiments on biological effects of fast neutron radiation. These data and results of cell kinetic studies on experimental animal tumours with regard to natural growth, effects of radiation and repopulation, indicate that mathematical models might be constructed by which the total effect of various dose fractionation schemes can be predicted although as yet with a limited accuracy. If adequate data on the effects in dose-limiting normal tissues were also available, optimal dose fractionation schemes could be calculated.

Unfortunately, we are still far from such a situation with regard to human tumours, where even for a group with similar histological classification a considerable variation has been shown with regard to growth rate, fraction of proliferative cells and percentage cell loss, as well as with respect to their radiosensitivity and curability. Clinical research should concentrate more on cellular criteria required to make radiation responses more predictable in the clinical situation.

With the introduction of fast neutrons in clinical radiotherapy, basic data on the cellular level in human tumours are certainly required in order to obtain a rational approach to an optimum dose and dose fractionation schedule. More than half a century of empirical studies in X-ray therapy has led to treatment schemes for most of the solid tumours, in which about 1000 rad is applied each week in 3, 5 or 6 equal fractions to a total dose that is infrequently outside the range of 5500–7000 rad for relatively limited volumes. The number of fractions per week depends strongly on circumstantial factors, like the length of the free week-end or efficient use of the apparatus. One is reluctant to envisage the possibility that another fifty years would be required to establish the treatment schemes necessary to exploit possible advantages of fast neutrons in an optimal way. It has to be acknowledged in this respect, that a treatment scheme based on the conventional schemes for X-rays, only taking into account an estimated RBE factor for one or two normal tissues, is but one of the many possibilities for an optimal use of neutrons. Consideration of the most important factors which cause differences in biological dose–effect relationships for fast neutrons as compared with X-rays, namely RBE variation as a function of the dose, a smaller influence of repair of sublethal damage, and a smaller oxygen enhancement ratio, can lead to the suggestion that quite different fractionation

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regimens might be required to exploit advantages of fast neutrons most effectively. This implies that even if with conventional daily treatments, improvements in curative results were not demonstrated, fast neutrons might still have a potential advantage. In addition, if with conventional fractionation of fast neutrons improvements are demonstrated, as the early data presented by Dr. Catterall indicate, we would still be faced with the requirement to design, on the basis of scientific data, other dose fractionation schemes to be tested with respect to further gains to be attained for sufficiently large groups of patients and different types of tumours.

Clinical trials with a considerable number of patients are obviously required to answer the various questions with respect to fractionation in neutron radiotherapy, such as

(*a*) What is the maximum tolerance dose for different areas of the body and which adverse effects occur?

(*b*) For what types of tumours and locations are neutrons most likely to provide an advantage over X-rays?

(*c*) Are fractionation schemes other than equal doses per fraction beneficial?

(*d*) Can combinations of fast neutrons and X-rays partly eliminate some disadvantages of fast neutrons (e.g. unfavourable depth dose and collimation)?

Radiotherapists starting with fast neutrons should aim at answering these questions as soon as possible for a number of reasons:

(1) The application of fast neutrons requires considerable investment of money and time;

(2) If hazardous and unsuccessful, neutrons should be eliminated from radiotherapeutical practice to protect future generations of patients and prevent waste of investments;

(3) If beneficial for specific types of tumours, fast neutrons should rapidly be made available for as many patients as possible.

In addition to the few radiotherapy centres which have already reported results of patient treatments, a larger number of centres will soon begin with the application of fast neutrons to patients. Nevertheless, the total number of patients to be treated with neutrons will remain rather low in the coming several years and if each centre works independently, it may take many years before definite conclusions can be drawn. Therefore, it is necessary to aim at international agreement, not only with respect to the description of physical characteristics of the neutron beams, dosimetry and collimation, but also with respect to the dose, dose fractionation and the criteria for evaluation of tumour responses and of late normal tissue reactions.

The need for international cooperative studies can be shown on the basis of data discussed by Withers *et al.* [1]. For instance, for a demonstration of an increase in local control from 30 to 40%, about 100 patients in each group treated with neutrons and X-rays, respectively, would be required. Even for a tumour that occurs relatively frequently, e.g. squamous cell carcinoma of the uterine cervix, a large centre will only have about 40–50 new patients in stage III per year. Commonly, a proportion of all patients does not fit all criteria for selection and consequently 4–5 years are required to enter enough patients in the trial, while a total of 7–8 years including observation will be necessary before definitive evaluation is possible. For tumours which occur less frequently, e.g. soft tissue sarcomas, much longer times will be required.

Another factor which makes international cooperation necessary is the fact that in most centres only a limited number of patients can be treated by the neutron generator available, either because of a low output or because the generators can only be used part-time.

Although the various factors discussed indicate that international cooperation is required, some problems related to such studies should not be ignored. For the controls, the beam energies and treatment schedules employed in various centres are not the same for conventional irradiations. It also remains to be investigated whether differences in neutron energy between various centres will not render an otherwise adequate comparison ambiguous. Finally, even if the neutron energy is the same for two centres, differences in collimators etc. might cause different gamma-ray contributions. Notwithstanding these problems, however, it appears necessary that the centres in Europe, North-America and Japan, that are going to apply fast neutrons in the coming years, should pool their resources.

As a first initiative in international cooperation with respect to fast neutron radiotherapy, a Fast Neutron Radiotherapy Project Group has been started, under the sponsorship of the European Organization for Research on Treatment of Cancer (EORTC). This organization has already experience with various groups coordinating clinical trials, e.g. on combined radio and chemotherapy and on radiotherapy. Close contacts with similar groups in the USA would clearly be in the interest of all centres involved.

REFERENCE

1. 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).

Europ. J. Cancer Vol. 10, pp. 387–390. Pergamon Press 1974. Printed in Great Britain.

Discussion on Plans for Fast Neutron and Negative Pion Radiotherapy at Various Centres*

W. L. CALDWELL (Madison, Wisconsin, U.S.A.): *The Wisconsin Society of Radiation Oncologists' fast neutron treatment facility*

BECAUSE in the near future a clinical "gas target D–T fast neutron generator" is likely to be available, the Wisconsin Society of Radiation Oncologists has discussed the problem of how such a unit can be used optimally by cooperating physicians for the benefit of the 4·5–5 million people of the State of Wisconsin and bordering communities of adjoining states.

An adequate number of patients must be available for treatment not only to allow prospective evaluation of treatment results, but also to provide adequate service income to at least partially meet the expenses involved in maintaining such a facility. With this in mind the Fast Neutron Treatment Center must be near the centre of the serviced population at a location which is readily accessible by major roadways.

As in most parts of the United States there is an increasing availability of well trained and full time radiation oncologists to provide the radiotherapy services in Winsconsin. Recently guidelines for the practice of radiotherapy within the state have been established [1]. Within the next two to three years it is anticipated that most of the radiotherapy practiced within the state will be by members of the Wisconsin Society of Radiation Oncologists, hence most patients suitable for treatment with fast neutrons will be available for trials at the Fast Neutron Treatment Center. Typical patients to be considered in protocols include those with certain tumours of the head and neck, brain, bladder, cervix, soft tissue and bones, prostate, and possibly the oesophagus and lung. The value of fast neutrons even in common palliative situations might be evaluated; e.g. for the management of intracranial metastases.

Present plans call for the centre to be headed by an experienced radiotherapist director. In addition there will be a permanent staff including physicists, a dosimetrist, radiotherapy technologists, a nurse, a statistician, and a secretarial staff. Biologic investigations will probably be accomplished on a comparable investigational machine at the University of Wisconsin, hence no radiobiologist will be on site at the Center.

It seems important that the various society members be actively involved in the Center's activities so that they will be aware of the care their patients are receiving and be apprised of the responses and reactions resulting for tumours at the various sites. Participating members of the Wisconsin Society of Radiation Oncologists will visit the Center every three weeks for an entire day. At such time he will see patients from his as well as from other institutes. In addition, patients from his institute will be seen in follow-up on his visiting days. This will allow continuity of care for the patient by both the director of the Center and the participating member.

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*Full address of contributors to the discussion can be found in the list of participants to be published in Vo 10, No. 6, on pp. 393 to 398.

REFERENCE

1. P. C. NUTT and P. C. TRACY, Standards and guidelines for a radiotherapy network in Wisconsin. *Wis. med. J.* **72,** 19 (1973).

S. B. CURTIS (Berkeley, Calif., U.S.A.): *Plans for the high-energy, heavy-ion facility (BEVALAC) at Berkeley*

THE Berkeley HILAC and Bevatron are presently being joined via a beam-transport line, so that heavy-ion beams accelerated at the HILAC can be injected into the Bevatron for additional acceleration to high energy. The high-energy, heavy-ion mode of operation is designated BEVALAC. The beam energy, after full acceleration of any ion from ^{4}He to ^{84}Kr, can be chosen within the range 100–2500 MeV/amu. Table 1 gives estimated average dose rates at extraction with $\pm 10\%$ dose uniformity over an area of 100 cm^2. The dose rate is inversely proportional to the area over which uniformity is desired. The initial energy of each beam was chosen such that the ion range would be 10 cm of water. If rapid scanning of a narrow beam over the area is assumed, the dose rates could be increased by a factor of four to five, since only the central 20% of a gaussianly distributed beam is used in estimating the dose rates in the Table. The LET_∞ and dose rate in the Bragg peak region will be considerably higher than the values in the Table, which are quoted for the initial portion of the dose-vs-depth curve. For instance, calculations of LET distributions in the Bragg peaks of the ^{14}N, ^{16}O and ^{20}Ne beams yield values in the 100 keV/μm region and above [1].

Presently an experimental program is in progress in radiobiology [2], utilizing Bevatron beams of carbon, nitrogen and oxygen ions of initial energy 260 MeV/amu at lower dose rates (up to rad/min over a cross sectional area of one cm^2).

REFERENCES

1. S. B. CURTIS, Calculated LET spectra from heavy primaries and secondaries in stopping heavy-ion beams in water. *Presented at 21st Meeting of the Radiation Research Society*, St. Louis (1973).
2. *Radiobiological Experiments Using Accelerated Heavy Ions at the Bevatron.* Lawrence Berkeley Laboratory Report No. 2016, Berkeley, Calif. (1973).

H. D. FRANKE (Hamburg, Germany): *Plans for neutron radiotherapy in the Radiotherapy Department of the University Hospital Hamburg-Eppendorf*

THE CLINICAL neutron therapy facility at Hamburg is now installed. The essential features are described in a separate paper in these proceedings [1], as well as the measuring methods [2, 3]. The neutron therapy equipment is specified for our clinical purposes and can be used for arc and multiple-field therapy. The dose rate will be approximately between 5–10 rad/min at the site of the tumour. We have started with our dosimetry program and hope to be able to begin the treatment of patients in the first half of 1974.

Table 1. *Projected dose rates in initial portion of Bragg curve for heavy-ion beams from the BEVALAC*

Ion	Initial energy for 10 cm range in H_2O (MeV/amu)	Initial LET_∞ (keV/μm)	Estimated average* flux (ions/min)	Estimated average† dose rate over 100 cm^2 uniformity $\pm$ 10% (rad/min)
^{4}He	120	2·6	3×10^{11}	250
^{6}Li	150	5	3×10^{11}	480
^{10}B	200	11	3×10^{11}	1000
^{12}C	230	15	6×10^{11}	3000
^{14}N	250	19	3×10^{11}	1800
^{16}O	270	24	3×10^{11}	2300
^{20}Ne	300	36	10^{11}	1150
^{40}Ar	400	100	5×10^{9}	160
^{84}Kr	600	340	5×10^{5}	0·05

*Ions are accelerated in pulses with spill times up to 1 sec in duration; the pulse rate can be varied between 10 and 17 pulses/min. The above values are calculated assuming 10 pulses/min.

†Dose rates, averaged over a one second beam spill time, will be six times larger.

We plan to begin with the treatment of palpable lymph node metastases in different sizes arising from differentiated tumours (adenocarcinoma, melanoma, etc.) and to compare the effect of telecobalt and fast neutrons. We intend to obtain quantitative data for single and fractionated doses by comparing the clinical with the histological tumour response. For these examinations we need the same dose distribution using telecobalt or neutrons. Afterwards we plan to investigate the effect of neutrons and telecobalt on lung metastases from different tumours, also with the same dose distribution between both radiations. We will analyze effects on different tumours as well as on different normal tissues (skin, lung-tissue, etc.). At the same time we intend to observe in special tumours the effect of neutrons on cell synchronization, on blood supply and the oxygenation. According to the fractionation scheme used at Hammersmith Hospital [4], time variation of the dose application should be investigated. With new data about the influence of fast neutrons on changes in oxygenation of tumours, it might be possible to irradiate at certain times of the treatment course with neutrons, for example, at the beginning and at the end. Depending on the degree of oxygenation we could use hyperbaric oxygen in the midst of the radiation period [5]. Later on irradiations of deep-seated tumours will be carried out.

REFERENCES

1. H. D. Franke, M. R. Cleland and B. F. Offermann, Neutron therapy facility at the Radiotherapy Department of the University Hospital Hamburg–Eppendorf. *Europ. J. Cancer* **10,** 256 (1974).
2. M. R. Cleland and J. Ryan, Neutron dosimetry system for routine treatment control. *Europ. J. Cancer* **10,** 317 (1974).
3. M. Bormann, R. Schmidt, R. Sittig and A. Hess, Energy distribution and depth doses of D–T neutrons in a tissue-equivalent phantom by activation threshold detectors. *Europ. J. Cancer* **10,** 316 (1974).
4. M. Catterall, Clinical experience with fast neutrons from the Medical Research Council's cyclotron at Hammersmith Hospital. *Europ. J. Cancer* **7,** 227 (1971).
5. H. D. Franke, Die Anwendung strahlensensibilisierender Substanzen in der Strahlentherapie. *Strahlentherapie* **143,** 296 (1972).

M. M. Kligerman (Albuquerque, New Mexico, U.S.A.) and D. F. Petersen (Los Alamos, New Mexico, U.S.A.): *Program leading to clinical trials at LAMPF*

Clinical protocols have been completed for trials of pion radiotherapy. These include those tumours which are currently poorly managed by any method such as tumours of the oesophagus and pancreas, and those in which results are poor in the advanced stages such as stage III and IV cancers of the head and neck, and inoperable cancer of the rectum. If the current cure rate is below 5–7%, controls with low-LET radiation will be omitted. The dose–time relationship will be that estimated to be equivalent to 7000 rad in seven weeks of low-LET radiation. Five treatments each week are planned. This should give maximum opportunity for normal cell repopulation as well as permit observation of the acute reaction of the normal tissues.

A low intensity channel is scheduled to operate in August, 1973. The biomedical beam line, which is planned to deliver a minimum of 40 rad/min to a treatment volume $15 \times 15 \times 15$ cm, is scheduled to be operational in September/October, 1973. Since the intensity of the beam will be kept low initially in order to permit tuning and minor structural changes, priorities for the preclinical biology studies are established suitably within the intensity range available.

The earliest cellular studies which will be carried out with peak dose rates of 2–10 rad/min will be repeated subsequently at higher intensities. The control radiation will be 250 kV X-rays. This is a specifically directed program which excludes experiments of biological interest if they are not required in the sequence leading to early clinical trial. However, time is planned for members of the LAMPF Users Group to conduct biological experiments not related directly to the clinical trial, if approved by appropriate review bodies.

Because of the length of time required to observe results, it was intended to start with animal experiments to establish long term tolerance of such normal structures as the skin, kidney, colon, spinal cord, heart and lungs. The initial low beam intensity makes this impractical. Therefore, initially, depth dose biological effect curves will be established in the peak and plateau regions using CHO, T1, and L5178Y lymphoma cells. Fractionation studies with these three cell lines will be carried out. This will be followed by a study on spleen colony formation as an example of comparative effects in a clonogenic system.

With increased output studies of effects in intestinal crypt will be undertaken. This will be followed by acute and long term effects on skin (pig and mouse foot), kidney, spinal cord

and lung. Data on kidney and lung using 250 kV X-rays are already well established and therefore these received priority for investigation. The effect of altitude, anaesthesia and immobilization on the P_{O_2} is under investigation. As output further increases, transplantable tumours (two types) in mice and spontaneous tumours in dogs will be studied.

When an appropriate amount of biological data are obtained, human radiobiology will be started looking at skin response, and establishing biological effect curves for multiple skin nodules, multiple superficial lymph node metastases, and multiple pulmonary metastases.

R. MORRISON (London, U.K.): *The planning of future trials of neutron therapy*

IN PLANNING future work and clinical trials to assess the value of neutron therapy and to determine the optimum method of applying this radiation, it is suggested that the protocol of future trials should be planned with two objectives.

(1) To solve a specific problem at each centre at which the trial is being conducted;

(2) To resolve general questions such as the effect of dosage, the effect of changes in fractionation, the value of combining low- and high-LET radiation, etc.

These questions could not be solved at one treatment centre, because of the magnitude of the trials involved and the number of patients required. They must be planned in collaboration with a number of centres. Multi-centre trials would also be required to evaluate the therapy for diseases that have a low incidence, for example for salivary gland tumours or fibrosarcomata where the preliminary results are encouraging.

In the present comparative clinical trial at Hammersmith being carried out by Dr. Catterall a neutron dose of 1440 rad is given in 12 fractions. This will tell us the local cure rate and the complication rate at the different sites of head and neck cancer. The optimum dose or fractionation is not known, however. It is suggested that a similar trial might be carried out on cancers at the same sites using a similar fractionation regime but changing the dose, say by 5–10%. The data obtained from a number of studies would provide information which could be used to construct a graph relating dose with tumour control rate and with complication rate. Similar investigations should be done using supervoltage X-rays. Data which is available about dosage with X-rays is scanty but suggests that both the tumour control rate and the complication rate rise rapidly beyond a certain critical dose level. It is suggested that it is only by having this sort of information that a full evaluation of neutron therapy can be made.

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Concluding Remarks on the Application of Fast Neutrons in Radiotherapy

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Comparison of the papers and discussions of the first and the second international meeting on "Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy" shows that considerable progress has been made with respect to various areas. Most important is the conclusion that at least for some types of tumours clinically favourable responses have been obtained in comparison with those expected from treatments with conventional radiations. Further progress from clinical applications can certainly be expected in the near future as more centres start treatments and follow up periods increase in length. It is essential that all centres maintain in close contacts and cooperate to obtain quantitative data on reactions of normal tissues and tumours as rapidly as possible.

With respect to the radiobiological aspects, it has become increasingly clear that beneficial results from fast neutrons do not depend only on the low OER and the presence of hypoxic cells in tumours, but that differences with regard to the influence of repair of sublethal damage among normal tissues and among tumours, and the reduction of this influence in the case of neutrons, might play an equally important role. The discussion of other factors e.g. repopulation by proliferation of cells in normal tissues as well as in tumours and variation of radiosensitivity with stage in the cell cycle have shown the enormous complexity of the problems involved in the application of radiobiological data to clinical radiotherapy. More and more accurate experimental data on responses of tumours and normal tissues, e.g. lung, spinal cord, and intestine, are required for fractionated treatments, to provide a basis for the clinical applications of fast neutrons.

With respect to dosimetrical aspects, considerable progress has been made by international cooperation, but more data are certainly required on the dose from scattered neutrons which have lower energies than those of the primary beam and consequently have larger RBE values. The contribution from scattered neutrons and from gamma rays to the total dose in the area to be irradiated as well as in the penumbra, will depend on the type of collimator, the size of the field and the thickness of the patient. Thus for each neutron generator considerable dosimetric information will have to be obtained separately.

A discussion of SI units showed that many participants want to retain the units rad and curie as special units.

With respect to neutron generators for radiotherapy, it appears that the development of various accelerators using the $^{3}H(d,n)^{4}He$ reaction to produce 15 MeV neutrons, has not yet proceeded to the stage where a constant dose rate of 10 rad/min at 1 metre from the target during periods of the order of 100 hours is attained routinely. With cyclotrons, beams of adequate dose rates can be produced and until now most patients have been treated with these beams, despite the absence of beam direction flexibility.

Finally it should be noted that the knowledge to be obtained from clinical applications of fast neutrons in close contact and cooperation between various centres will also be of great importance for the application of negative pions and heavy ions which are expected to

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have similar biological properties while better collimation and depth dose characteristics are attained. The data reported until now indicate that definite gains can be obtained, but further progress can only be made by a cautious approach, based on radiobiological insights and careful clinical observations, preferably in as quantitative terms as possible.

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List of Participants at the 2nd Meeting on Fundamental and Practical Aspects of the Application of Fast Neutrons in Clinical Radiotherapy

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Comparison of Adriamycin with the DNA-Adriamycin Complex in Chemotherapy of L1210 Leukemia*

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Abstract—*The effect of adriamycin and a complex DNA–adriamycin was compared in normal mice and in mice with leukemia L_{1210}. The DNA–adriamycin was found to be less toxic than adriamycin, especially during the first few days of administration. Increased therapeutic effectiveness was observed in one type of leukemia L_{1210} which was obtained from another laboratory and has a better prognosis than the L_{1210} maintained in our laboratory and on which no therapeutic enhancement of the DNA–adriamycin complex was demonstrated.*

The incidental finding that L_{1210} leukemia from different strains and laboratories may show different responses to the same treatment invites precaution in the interpretation of comparative studies with this model.

INTRODUCTION

RECENTLY Trouet *et al.* have shown, in the case of daunorubicin, that complexing with DNA abolished the bacteriostatic *in vitro* effect of the pure drug, while if lysosomal extract was added to the culture medium, the bacteriostatic activity was restored presumably because the DNA moiety of the complex molecule was destroyed by the lysosomal enzymes [1]. Trouet also found that DNA–daunorubicin, tested on a variety of experimental tumors in animals was less toxic and could be used at higher and more active doses than daunorubicin alone. This was attributed to absorption of the complex by pinocytosis preferentially by cancer cells and release of the free daunorubicin inside of the cell by lysosomal action [1].

In order to investigate the application of this concept to another chemotherapeutic agent, we have compared the therapeutic effectiveness and subacute toxicity of free adriamycin and the DNA adriamycin complex in normal and tumor-bearing mice. In the course of these experiments, we had the opportunity to make observations of the comparative therapeutic effectiveness and toxicity of the 2 agents.

These observations are the subject of the present report.

MATERIAL AND METHODS

Adriamycin was supplied by Farmitalia Benelux. DNA type V Sigma Chemical Corporation, St. Louis, Missouri, U.S.A., was prepared in and obtained from Dr. Trouet's laboratory. The DNA solution, titrating 4·68 mg per ml of water, was sterilized by heating at 120°C before use.

Experimental animals and tumors

BDF_1 mice ($DBA/2 \times C_{57}B1$) and DBA/2 mice (supplied by the Charles River Laboratory) were used. The leukemia L_{1210} used for inoculation is maintained in this laboratory by serial ascitic inoculation in DBA/2 mice: this leukemia was originally obtained from Wodinsky

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Table 1. *Comparison of the effect of DNA–adriamycin and adriamycin on the life span of DBA/2 mice bearing* L_{1210} *leukemia*

	Leukemia L_{1210}T was obtained from Dr. Trouet *et al.* [1]				Leukemia L_{1210}B was our serially maintained leukemia at the Institut Jules Bordet (supplied by Dr. Wodinsky, Arthur Little Co.)			
	DBA/2 mice with L_{1210}T				DBA/2 mice with L_{1210}B			
	Adriamycin		DNA–adriamycin		Adriamycin		DNA–adriamycin	
Dose mg/kg	Life span*	Number of survivors after 30 days	Life span*	Number of survivors after 30 days	Life span*	Number of survivors after 30 days	Life span*	Number of survivors after 30 days
6	83	0	216	1	42	0	83	0
5	141	0	216	0	42	0	50	0
3	92	0	125	0	33	0	8	0

DBA/2 male mice (18–24 g) were inoculated i.v. (day 0) with 10^5 leukemic cells from stock DBA/2 mice bearing ascitic L_{1210}.

Daily treatment was given from day 1 to day 5.

*Increase in percentage.

Table 2. *Comparison of the effect of DNA–adriamycin and adriamycin on the life span of* BDF_1 *mice bearing* L_{1210} *leukemia*

	Leukemia L_{1210}T (see Table 1)					Leukemia L_{1210}B (see Table 1)			
	BDF_1 with leukemia T					BDF_1 with leukemia B			
	Adriamycin		DNA–adriamycin			Adriamycin		DNA–adriamycin	
Dose mg/kg	Life span*	Number of survivors after 30 days	Life span*	Number of survivors after 30 days	Dose	Life span*	Number of survivors after 30 days	Life span*	Number of survivors after 30 days
6	0	0	6	0	6	—	0	6	0
5	0	0	12	0	3	12	0	12	0
3	50	0	100	0	1·5	0	0	0	0

BDF_1 male mice (23–28 g) were inoculated i.v. day 0, with 10^5 leukemic cells from stock DBA/2 mice bearing ascites L_{1210}.

Daily treatment was given day 1–5.

*Increase in life span in percentage.

(Arthur D. Little Co.). For purposes of comparison as indicated in Tables 1 and 2, we also carried out experiments with a leukemia L_{1210} originally obtained from Trouet's laboratory [1] and maintained serially in our laboratory. In experiments in which the 2 types of leukemia were used, they are clearly identified by the letter T (Trouet) or B (Bordet). When there is no specification, the leukemia L_{1210} from Wodinsky (A. D. Little Co.) was used (Leukemia L_{1210}B).

In the present experiments, 10^5 leukemic cells in 0·1 ml of Hank's balanced solution were injected intravenously into BDF_1 and DBA/2 mice. Treatment was begun 24 hr after implantation of the leukemic cells and administered intravenously once a day for a total of 5 injections.

Preparation of the DNA–adriamycin complex: this was done by the method described previously [1] by dissolving separately the adriamycin and the DNA in isotonic sodium chloride solution. The adriamycin solution was added to the DNA solution under constant stirring. The molar ratio DNA mononucleotides/adriamycin of the final solution was 20; for one ml of the adriamycin solution containing 20 mg per ml there was 50 ml of the DNA solution containing 4·68 mg per ml. The mean molecular weight of one mononucleotide is considered to be 327 daltons.

Toxity of the DNA–adriamycin complex

was determined on BDF_1 and DBA/2 intact mice by the administration of 5 intravenous injections given on 5 consecutive days.

In our experiments, a loss of 4 g or more of body weight ascertained on the 5th day was considered as a sign of toxicity. The ratio of the median survival time of the test group to that of the control (T/C) also served as an index of toxicity. A ratio T/C smaller than or equal to 85/100 indicated definite toxity.

RESULTS

1. *Comparison of the antileukemic effect of DNA–adriamycin and of adriamycin*

This was carried out in animals with L_{1210} early leukemic from 2 sources, T and B. These 2 leukemias were inoculated into 2 strains of mice: DBA/2 and BDF/1 and survival studies conducted under treatment. The DBA/2 male mice weighing from 18 to 24 g were inoculated intravenously with 10^5 leukemic cells from stock DBA/2 mice bearing the ascitic L_{1210} leukemia. Thirteen mice were used in the test and 22 mice as control. The BDF_1 mice, weighing 23–28 g were treated identically. Thirteen mice were used in the test and 34 mice as controls (Table 2). The data show that the DNA–adriamycin complex was therapeutically more active at the doses used here than the adriamycin alone in the leukemia L_{1210} T; the difference between DNA–adriamycin and adriamycin alone was much less apparent on the leukemia$_{1210}$B (Table 1).

However, when BDF_1 hybrid mice were used (Table 2), no appreciable difference could be demonstrated between the therapeutic effectiveness of DNA–adriamycin and adriamycin alone. There was also no appreciable difference between the effects on the 2 types of leukemia L_{1210}.

2. *Toxicity of DNA–adriamycin v. adriamycin alone*

This was studied on intact DBA/2 and BDF_1 mice by administering in each case DNA–adriamycin to 10 mice and free adriamycin to 10 control mice. Figures 1 and 2 show the 10 days survival data after the administration of various dosages of free and combined adriamycin to the experimental groups of animals. For the same amount of adriamycin, survival was much increased in the DNA–adriamycin group and this increase was found to be more pronounced in DBA/2 than in BDF_1 mice (Figs. 1 and 2). However, this prolonged survival was much less apparent after 30 days

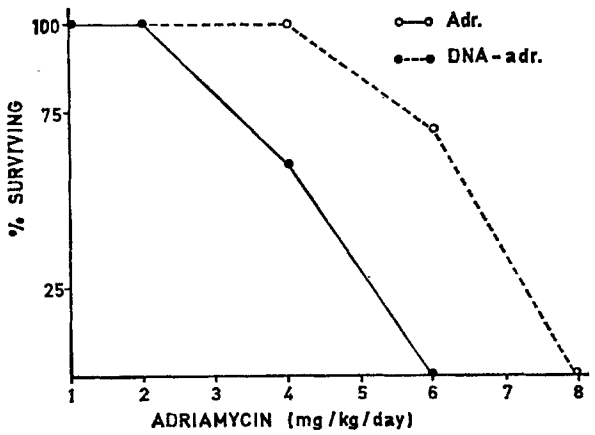


Fig. 1 Subacute toxicity of free adriamycin v. DNA adriamycin injected i.v. in BDF/$_1$ mice. Ten mice used for each dose. Results after 10 days.

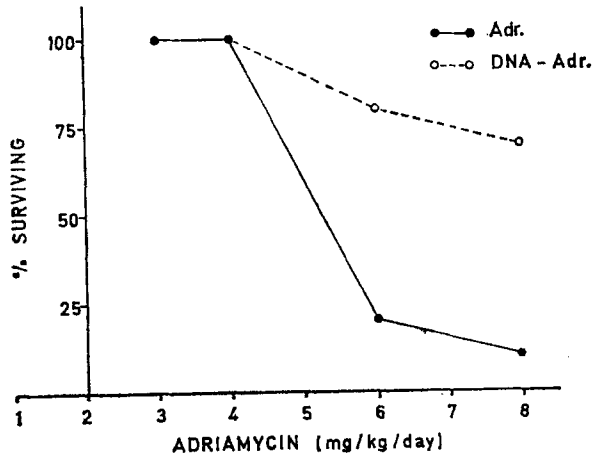


Fig. 2 Same as Fig. 1 except that DBA/$_2$ mice were used.

than after 10 days (Figs. 3 and 4) especially in DBA/2 mice. This increased survival was interpreted as a sign of reduced toxicity. However, this difference in toxicity between the free and combined form of adriamycin, when evaluated in leukemic mice on the 5th day, was expressed by changes in body weight only and did not correspond to an increased therapeutic effectiveness as evaluated by survival figures. This is seen in Table 3 which shows body weight changes and survival figures observed in leukemic mice treated either by free adriamycin or DNA–adriamycin. In these experiments, the leukemia L_{1210}B was exclusively used.

DISCUSSION

Our data show that the DNA–adriamycin complex was less toxic than the equivalent amount of free adriamycin on DBA/2 and BDF_1 mice if the toxicity is evaluated by changes of body weight. However, the difference in toxicity became attenuated after the first 10 days of observation. There is also the

*Table 3. Comparison of the therapeutic effect and toxicity of adriamycin and DNA–adriamycin in early leukemia in BDF/1 mice**

Number of L_{1210} cells inoculated: 10^5 per mouse					Number of L_{1210} cells inoculated: 10^5 per mouse				
	Adriamycin		DNA–adriamycin			Adriamycin		DNA–adriamycin	
Dose mg/kg	Body weight change (g on day 5)	T/C†	Body weight change (g on day 5)	T/C†	Dose mg/kg	Body weight change (g on day 5)	T/C†	Body weight change (g on day 5)	T/C†
7	−5·6	87	−3·4	100	7	−3·6	74	−2·3	84
6	−5·5	87	−3·3	106	6	−3·1	74	−1·6	84
3	−1·7	112	−1·6	112	4	−2·9	84	−1·0	105
1·5	−1·1	100	+0·6	100	2·5	−1·3	153	−1·2	137

*Male mice (23–28 g) inoculated by i.v. route on day 0. Treatment given daily from day 1 to day 5. $L_{1210}B$ used exclusively.
†T/C = median survival of treated mice/median survival of control, in percentage.

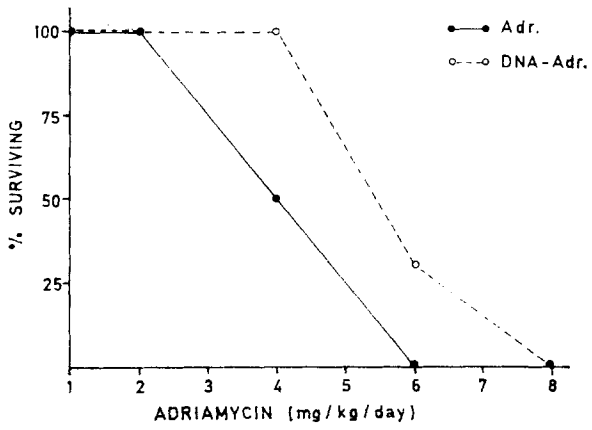


Fig. 3 Subacute toxicity of free adriamycin v. DNA–adriamycin injected i.v. in BDF/1 mice. Results after 30 days.

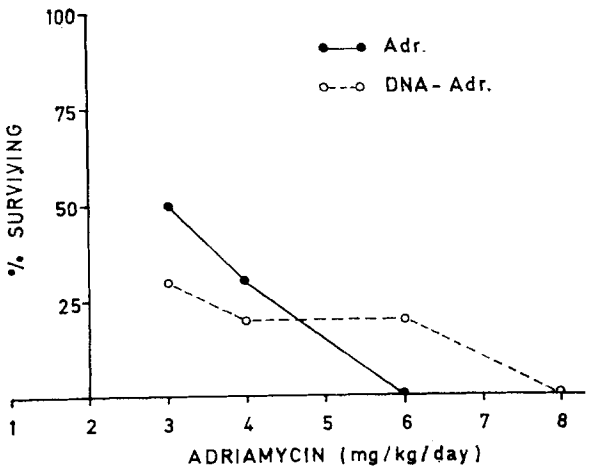


Fig. 4 Same as Fig. 3 except that DBA/2 mice were used.

fact that little or no difference in toxicity between the 2 forms of adriamycin administration was observed in leukemic mice of the same strains, especially in BDF_1 mice. In the mice with leukemia $L_{1210}B$, no improved therapeutic activity could be attributed to the complex as compared to adriamycin alone.

Although the data do not provide an explanation for these observations, there are 2 possible interpretations which can be envisaged although they remain purely hypothetical: (1) In Trouet's interpretation [1], the DNA–adriamycin complex in actually pynocytosed preferentially by leukemic cells present in the organism and it is conceivable that more adriamycin is liberated from the complex in leukemic than in intact animals. Since the reduction of toxicity is probably a function of the persistence of the complex in the organism, dissociation of the complex by lysosomal digestion in the leukemic cells and subsequent release of free adriamycin would explain the increased toxicity on DNA–adriamycin for leukemic as compared with normal mice.

Another hypothesis to be considered is that there could be differences in concentrations of DNA–ase in the serum of BDF_1 mice and of DBA/2 mice. A higher concentration of serum DNA–ase would tend to increase the toxicity. Furthermore, this DNA–ase serum concentration could vary in DBA/2 mice rendered leukemic by L_{1210} cells inoculation. There are data from Trouet supporting these 2 hypotheses. They are still preliminary, but might contribute to explaining the observed differences in toxicity and activity described in this communication. They will have to be confirmed and expanded before the real mechanism of action of DNA–adriamycin on mouse leukemia is understood.

The difference of effectiveness in favor of the DNA–adriamycin complex as compared to adriamycin alone was demonstrated only on mice bearing, of the 2 types of L_{1210} leukemia

tested here, the one most responsive to treatment. This appears clearly in Table 1: The leukemia $L_{1210}T$, which was much more responsive to adriamycin alone than the leukemia $L_{1210}B$ also showed a better response to the DNA–adriamycin complex than to adriamycin alone.

The reduced toxicity of the complex for the organism under the experimental conditions of this work combined with a maintained or even enhanced therapeutic effectiveness suggests the possibility of selective action against leukemic cells of DNA–adriamycin, similar to that described by Trouet for DNA–daunorubicin.

An incidental finding in this work is the observation that leukemia L_{1210} from different strains of mice and from different laboratories may differ markedly in prognosis as well as in response to treatment. Therefore, great precaution is indicated in the interpretation of therapeutic results obtained on this experimental model and care should be taken, in all comparative therapeutic studies, that an identical strain of L_{1210} is always used.

Acknowledgements—Our thanks are due Dr. Omar Karim of the Farmitalia Benelux for a generous supply of adriamycin.

REFERENCE

1. A. Trouet, D. Deprez-De Campeneere and C. de Duve, Chemotherapy through lysosomes with a DNA–daunorubicin complex. *Nature, New Biol.* **239,** 110, 1973.

Europ. J. Cancer Vol. 10, pp. 405–411. Pergamon Press 1974. Printed in Great Britain

Experimental Leukemia Chemotherapy with a "Lysosomotropic" Adriamycin-DNA Complex

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Abstract—*The complex formed between adriamycin and DNA possesses the physicochemical and* in vitro *biological characteristics of a lysosomotropic drug. Adriamycin is resistant to lysosomal hydrolases and binds firmly to high molecular weight DNA. When linked to DNA, adriamycin looses its properties of fluorescence, of dialysis and its antibiotic activity. Adriamycin can however be released from the complex and recovers all its properties after digestion of the DNA by lysosomal desoxyribonucleases.*

The adriamycin–DNA complex is two times less toxic than free adriamycin when given intraperitoneally or intravenously on five consecutive days in NMRI mice.

While two times less toxic than free adriamycin, adriamycin–DNA possesses higher chemotherapic efficiency against the L1210 Leukemia in DBA_2 mice. If one takes advantage of these two properties, adriamycin–DNA induces very high percentages of increase in life span in DBA_2 mice inoculated intravenously with L1210 cells and allows a great proportion of mice to survive longer than 30 days.

All these data are in favor of a lysosomotropic mode of action of adriamycin–DNA. According to this hypothesis, adriamycin–DNA penetrates only the cells possessing a significant endocytic activity. After degradation of DNA inside the lysosomes, adriamycin becomes free and exerts its toxic activity mostly on those cells, which like some tumor cells, possess besides an high endocytic activity an high mitotic rate. Many normal cells with an high mitotic rate or susceptible to the action of adriamycin but with a low endocytic activity, are in this way protected.

INTRODUCTION

IN A PREVIOUS paper [1] we defined the concept of lysosomotropic cancer chemotherapy and we presented its first practical application with daunorubicin–DNA.

If an antitumoral drug is firmly linked to an endocytizable and digestible carrier, it will penetrate only cells endowed with an high endocytic activity. After intralysosomal digestion of the carrier, the drug will become free to diffuse from the lysosomes and to reach other components or the extracellular space. Such a lysosomotropic drug will, theoretically, be less toxic than the corresponding free antitumor agent since its activity will be restricted to the cells, which like some tumor cells, possess simultaneously high endocytic and mitotic activities. Many normal cells possessing neither or only one of these activities will on the other hand be protected against unwanted toxic effects.

In this paper we present the biological,

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toxicological and chemotherapic properties of a lysosomotropic form of adriamycin obtained by formation of a complex with DNA. Adriamycin is structurally related to daunorubicin [2] but seems to possess a greater activity against solid tumors [3]. The clinical usefulness of adriamycin and of daunorubicin is however severely restricted by their high toxicity for the bone marrow and the heart muscle. Any form or derivative of adriamycin with a lesser degree of toxicity or an higher chemotherapic efficiency should therefore be of great interest in the treatment of the various forms of leukemia and solid tumors.

METHODS

Preparation of adriamycin–DNA complex

Adriamycin (kindly supplied by Farmitalia, Brussels) is dissolved in sterile distilled water to a concentration of 20 mg/ml. Herring sperm DNA (highly polymerized, Type VII, Sigma, St-Louis) is dissolved in sterile 0,15 M NaCl to a concentration of 2·34 mg/ml, autoclaved for 15 min at 120°C and cooled slowly. Unless otherwise stated, one volume of the adriamycin is then mixed to 100 volumes of DNA to form a solution of complex with a molar ratio of DNA mononucleotide to adriamycin equalling 20. In a few experiments different molar ratios were used.

Fluorescence assay of adriamycin

The fluorescence of adriamycin is measured in a Zeiss ZMF 4 spectrofluorometer. An activation wavelength of 485 nm and an emission wavelength of 580 nm are used.

Bacteriostatic assay

The antibiotic properties of adriamycin are assessed by adding increasing amounts of the drug in a volume of 0·5 ml to 4 ml of Bacto dextrose Broth (Difco) adjusted at pH 8 and containing 0·5 ml of a *Staphylococcus albus* suspension in exponential growth phase. Bacterial growth is estimated by turbidimetry at 660 nm after 4–5 hr of incubation at 37°C. The results are expressed in percentage of the optical density of a control culture incubated without drug. Each test is performed in triplicate and results given as means ± standard deviations.

Equilibrium dialysis assays

The binding parameters of adriamycin to DNA are determined by equilibrium dialysis carried out in 10 ml dialysis cells. DNA was held at a nucleotide molarity (average molecular weight of 327) of $3{\cdot}5 \times 10^{-3}$M and the concentration of adriamycin varied from $3{\cdot}5 \times 10^{-2}$M to $1{\cdot}4 \times 10^{-1}$M (DNA nucleotide/adriamycin ratio between 10 and 40). The free and complex drugs are dissolved in 0·15 M NaCl containing 0·01 M pH 7 phosphate buffer and dialysis performed in the dark at 37°C.

The concentration of the free drug in the external compartment is measured by fluorometry after varying times until equilibrium is reached (± 90 hr). Results are expressed according to the Scatchard formula: $r/m = K_{app}(B_{app}-r)$, where r is the ratio of bound adriamycin molecules to DNA nucleotides, m is the molar concentration of free adriamycin, K_{app} is the apparent binding constant and B_{app} is the apparent number of binding sites for adriamycin.

Digestion experiments

Adriamycin and adriamycin–DNA (both at the concentration of adriamycin of 50 μg/ml) are incubated for 72 hr at 37°C with a soluble lysosomal extract in a 0·05 M acetate buffer pH 4·5. The lysosomal extract is a soluble fraction obtained from purified rat liver lysosomes [4].

Dialysis experiments

Dialysis bags containing 1 mg adriamycin (free or as a DNA complex) and 6·2 mg of lysosomal extract proteins in 15 ml of pH 4·5 0·05 M acetate buffer, are stirred at 37°C in 50 ml of an 0·05 M pH 4·5 acetate buffer dialysis medium. At varying times the dialysis medium is removed and its free adriamycin content measured by fluorometry. Free and DNA complexed adriamycin are incubated in the same conditions but without lysosomal proteins.

Mice strains

In the toxicity experiments NMRI mice are used, while the chemotherapic activity of the adriamycin and adriamycin–DNA are tested on DBA/2 mice.

L1210 leukemia

L1210 leukemia was obtained in its intraperitoneal form from Dr. C. Gosse and J. Morizet at Villejuif (France). These cells are propagated since 1972 in our laboratory by intraperitoneal inoculation into DBA/2 mice. Chemotherapic tests are performed on DBA/2 mice inoculated intraperitoneally or intravenously with L1210 cells.

RESULTS

Resistance of adriamycin to lysosomal hydolases

As shown in Fig. 1 adriamycin retains all its antibiotic activity after incubation with a lysosomal extract at pH 4·5 and 37°C for 72 hr. Similar results were obtained after incubation at pH 3·5 and 5·5.

Physical and antibiotic properties of adriamycin–DNA

The equilibrium dialysis experiments (Fig. 2) indicate an apparent binding constant of adriamycin to DNA of $0{\cdot}41 \times 10^6\ M^{-1}$. DNA possesses on the other hand one adriamycin binding site for every 5 nucleotides (B_{app}: 0·19).

As shown by Calendi [5] for daunorubicin, addition of adriamycin to DNA induces a quenching of the fluorescence (Fig. 3) and a reduction of the dialysis (Fig. 4) properties of adriamycin. Simultaneously adriamycin looses its antibiotic activity (Fig. 6).

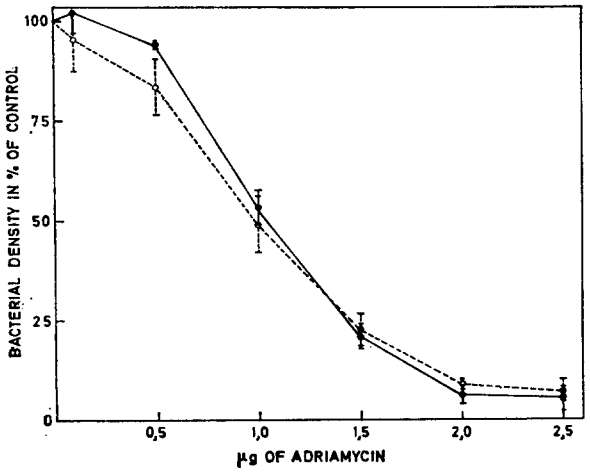


Fig. 1. Antibiotic activity of adriamycin after incubation with a lysosomal extract. ●—●, *adriamycin preincubated for 72 hr at 37°C with a soluble lysosomal fraction (0·75 mg of proteins/ml) at pH 4·5.* ○---○, *adriamycin preincubated in similar conditions but without lysosomal proteins.*

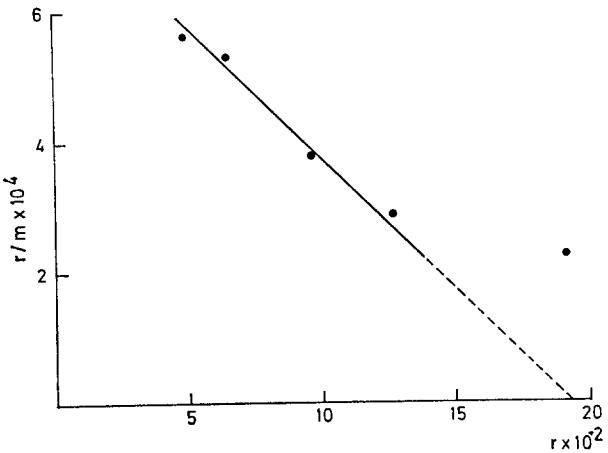


Fig. 2. Scatchard plot of the binding of adriamycin to herring sperm DNA

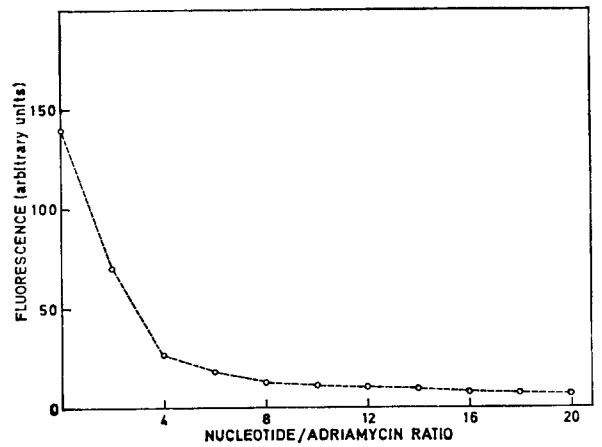


Fig. 3. Fluorescence of adriamycin and adriamycin–DNA at various nucleotide/adriamycin molecular ratios.

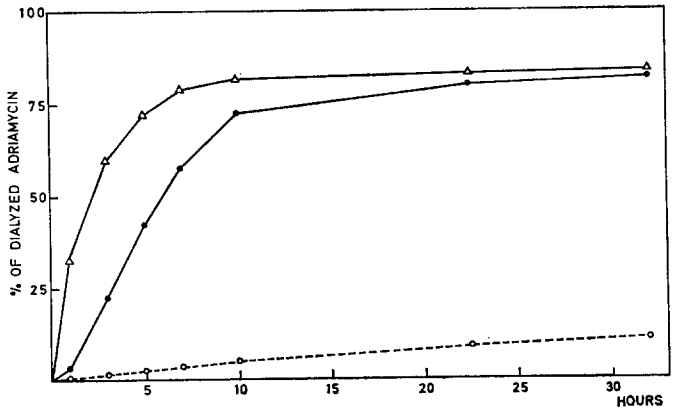


Fig. 4. Dialysis properties of adriamycin and adriamycin–DNA complex incubated at pH 4·5 and 37°C. △—△, *free adriamycin.* ○---○, *adriamycin–DNA complex.* ●—●, *adriamycin–DNA in presence of a soluble lysosomal fraction (0·45 mg of protein per ml).*

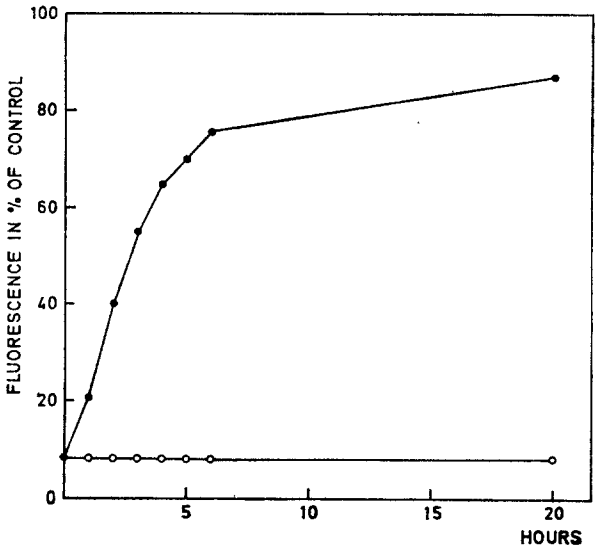


Fig. 5. Fluorescence of adriamycin–DNA during incubation with a lysosomal extract at pH 4·5. ●—●, adriamycin–DNA incubated with a soluble lysosomal fraction (0·45 mg of proteins per ml) at pH 4·5. ○—○, adriamycin–DNA incubated in similar conditions without lysosomal proteins.

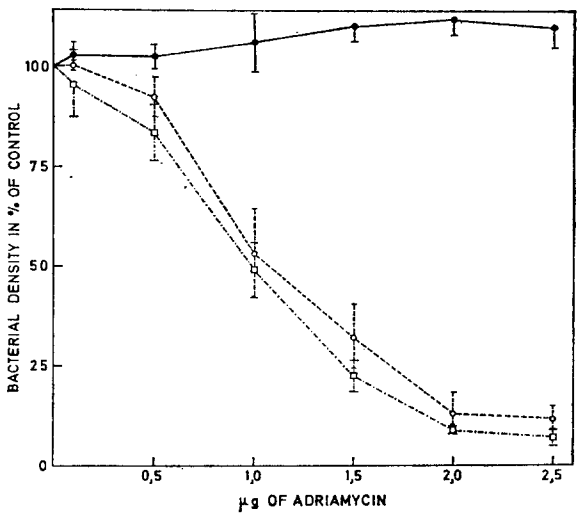


Fig. 6. Antibiotic activity of adriamycin–DNA after incubation with a lysosomal extract. ●—●, adriamycin–DNA preincubated for 72 hr at 37°C in pH 4·5 buffer. ○---○, adriamycin–DNA preincubated in the same condition with a soluble lysosomal fraction (0·65 mg of protein per ml). □-.-□, free adriamycin similarly incubated.

Incubation of adriamycin DNA with lysosomal hydrolases

The data of Figs. 4, 5 and 6 clearly indicate that incubation of adriamycin–DNA with a lysosomal extract at pH 4·5 restores at least 90% of the adriamycin properties of fluorescence, dialysis and bacteriostasis.

Toxicity of adriamycin–DNA in NMRI mice

The comparative toxicity of free and complex adriamycin was studied on the basis of the lethality, estimated after 90 days, of increasing amounts of drugs administered i.p. or i.v. for 5 consecutive days. As shown by Figs. 7 and 8, in these experimental conditions, the dosis inducing 50% lethality is more or less 2 times higher for adriamycin–DNA than for free adriamycin.

Chemotherapeutic activity of adriamycin DNA on L1210 leukemia in DBA_2 mice

The chemotherapic activity of free and complex adriamycin was studied on DBA/2 mice inoculated i.p. or i.v. with L1210 cells.

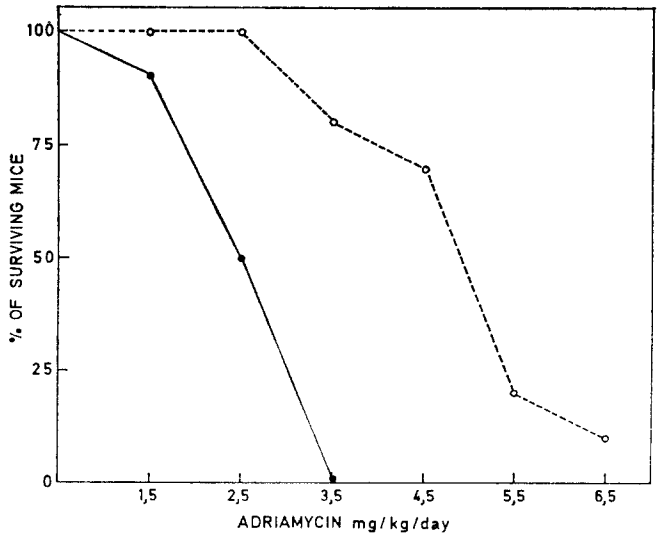


Fig. 7. Toxicity of adriamycin and adriamycin–DNA administered intraperitoneally. Series of 15 NMRI mice were injected i.p. on 5 consecutive days with variable doses of free or adriamycin–DNA. The percentage of animals surviving 90 days after the first injection is given in ordinate. ●—●, adriamycin. ○---○, adriamycin–DNA

In most tests we used the i.v. form of leukemia and we administered the drug by the same route, dealing in this way, with an experimental situation more closely related to the human leukemia and to its way of treatment. The results are summarized in Table 1.

DISCUSSION

Since the bacteriostatic activity of adriamycin is retained after incubation with a lysosomal extract, we can conclude that, if the antibiotic activity of adriamycin is related to the integrity of its molecular structure, adriamycin is resistant to lysosomal enzymes. Adriamycin is thus unlikely to be destroyed or modified during its stay in the lysosomal compartment of the cells *in vivo* and it thus complies with one of the most important *in vitro* criteria of a drug susceptible to be rendered lysosomotropic.

Adriamycin binds very tightly to DNA, the binding constant of $0{\cdot}41 \times 10^6 M^{-1}$ we measured

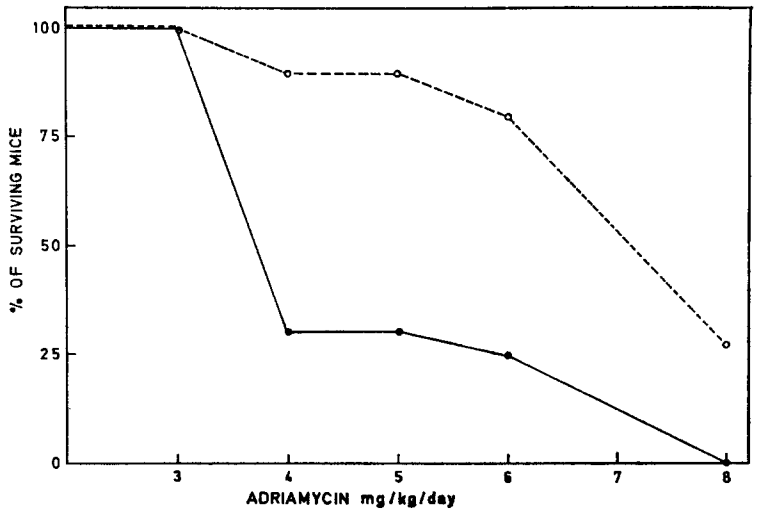


Fig. 8. Toxicity of adriamycin and adriamycin–DNA administered intravenously. Series of 15 NMRI mice were injected intravenously on 5 consecutive days with variable doses of free or adriamycin–DNA. The percentage of animals surviving 90 days after the first injection is given in ordinate. ●—●, *adriamycin.* ○---○, *adriamycin–DNA.*

Table 1. Chemotherapic activity of adriamycin and adriamycin–DNA

L1210 Leukemic cells		Drug			Median survival time (days)	% Increase in life span	Long term survivors (30 days)	Total number of mice
Number	Route of inoculation	Form	Route of administration	doses mg/kg per day				
10^4	i.p.	AM	i.p.	1	20	100	3	10
10^4	i.p.	AM–DNA	i.p.	1	27	170	4	10
10^4	i.p.	—	—	—	10	—	—	9
10^4	i.v.	AM	i.v.	2, 5	13	62	0	15
10^4	i.v.	AM–DNA	i.v.	2, 5	15	87	0	15
10^4	i.v.	AM	i.v.	4	15, 5	94	0	13
10^4	i.v.	AM–DNA	i.v.	4	18, 5	131	2	14
10^4	i.v.	AM	i.v.	5	14, 5	81	0	14
10^4	i.v.	AM–DNA	i.v.	5	23, 5	194	4	15
10^4	i.v.	AM	i.v.	6	8	0	0	13
10^4	i.v.	AM–DNA	i.v.	6	60	650	8	12
10^4	i.v.	—	—	—	8	—	—	29
10^5	i.v.	AM	i.v.	5	16	167	0	10
10^5	i.v.	AM–DNA	i.v.	5	18, 5	208	0	8
10^5	i.v.	AM	i.v.	6	16	167	0	11
10^5	i.v.	AM–DNA	i.v.	6	30, 5	408	5	10
10^5	i.v.	—	—	—	6	—	—	10

Drugs (AM: adriamycin; AM–DNA: adriamycin–DNA) were administered on 5 consecutive days starting on day 1. Mice were inoculated on day 0 with leukemic cells harvested from a 6 to 8 days old ascitic form of L1210 leukemia.

is however inferior to that of $2{\cdot}8 \times 10^6$ M^{-1} obtained by Zunino *et al.* [6] with calf thymus DNA. This discrepancy can be explained by the fact that our equilibrium measurements were performed at 37°C instead of 20°C, in presence of 0·15 M NaCl instead of 0·1 M Tris HCl, and that our DNA was of herring sperm origin.

Herring sperm DNA possesses apparently one adriamycin binding site for every five nucleotides and this result is confirmed by the fluorescence quenching data. These experiments indicate indeed that a sharp increase in quenching is obtained when the nucleotide adriamycin ratio raises from 1 to 4, and that at values higher than 10, the quenching is only slightly affected by an excess of nucleotides. We estimate that there are between one and two binding sites for every 10 nucleotides.

As a consequence and in order to have a nucleotide excess of at least 2 times we used in all our subsequent studies adriamycin–DNA complexes with a nucleotide–adriamycin ratio of 20.

When linked to DNA, adriamycin looses its dialysis properties and bacteriostatic activity suggesting that the complexed drug is unable to cross the bacterial walls and membranes. The physical and biological activities of adriamycin can however be restored after incubation of the complex with lysosomal hydrolases. This is most probably due to the digestion of the DNA carrier by lysosomal DNAses since the same liberating effect can be obtained with purified pancreatic or splenic DNAses.

From the *in vitro* experiments we can conclude that adriamycin–DNA has all the characteristics of a lysosomotropic drug. While adriamycin is resistant to lysosomal hydrolases it looses its biological activity when tightly bound to a macromolecular DNA carrier. This carrier can be digested by lysosomal enzymes and release adriamycin in a free form with all its biological activities.

Adriamycin–DNA is less toxic than the free drug when administered intravenously as well as intraperitoneally on five consecutive days in NMRI mice. The lower toxicity of adriamycin–DNA is in accordance with the lysosomotropic hypothesis and can be explained by a diminished sensitivity to the complex of normal cells possessing an high mitotic rate but a low endocytic activity. One major difference appears in these results between daunorubicin–DNA and adriamycin–DNA. While daunorubicin–DNA is less toxic than free daunorubicin when given intraperitoneally it is, in contrast with adriamycin–DNA, as toxic as free daunorubicin when administered intravenously. We have no explanation today for this difference in behavior between adriamycin–DNA and daunorubicin–DNA.

The results summarized in Table 1 indicate first that at non toxic (with regard to free adriamycin) doses (1 mg/kg per day intraperitoneally, or 2·5 mg/kg per day intravenously) the complex is significantly more active than the free drug. This higher chemotherapic efficiency can be explained by an higher sustained plasma level of the complex, by an higher intracellular concentration of the complex due to the high endocytic activity of the tumor cells or by both effects. The high chemotherapic efficiency of adriamycin–DNA becomes however much more striking at higher doses which are toxic with regard to the free drug. Very high increases in life span are then obtained with an high percentage of leukemic animals surviving more than 30 days and which can be considered to be cured. This result is confirmed with an high initial leukemic cells load of 10^5. The per cent increase in life span exceeds very much these obtained in the same experimental system with daunorubicin–DNA [7].

In conclusion, adriamycin–DNA is less toxic and more effective than the free adriamycin in L1210 leukemic DBA_2 mice. Our physicochemical and biological data are in favor of a lysosomotropic mode of action of adriamycin–DNA. In the experimental system used, adriamycin–DNA seems on the other hand to possess a much higher chemotherapic efficiency than daunorubicin–DNA.

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REFERENCES

1. A. Trouet, D. Deprez-de Campeneere and C. de Duve, Chemotherapy through lysosomes with a DNA–daunorubicin complex. *Nature, New Biol.* **239,** 110 (1972).
2. F. Arcamone, G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol and C. Spalla, Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius var. caesius. Biotechnol. Bioengin* **11,** 1101 (1969).

3. 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.* **30,** 2572 (1970).
4. A. Trouet, Immunisation de lapins par des lysosomes hepatiques de rats traites au Triton WR-1339. *Arch. Intern. Physiol. Biochim.* **72,** 698 (1964).
5. E. Calendi, A. Di Marco, M. Reggiani, B. Scarpinato and L. Valentini, On physicochemical interactions between daunomycin and nucleic acids. *Biochim. biophys. Acta* **103,** 25 (1965).
6. F. Zunino, R. Gambetta, A. Di Marco and A. Zalcara, Interaction of daunomycin and its derivatives with DNA. *Biochim. biophys. Acta* **277,** 489 (1972).
7. C. de Duve and A. Trouet, Lysosomes and lysosomotropic drugs in host-parasite relationship. In *Non-Specific Factors Influencing Host Resistance* (Edited by W. Braun and J. Ungar) pp. 153 (1973). S. Karger, Basel.

Europ. J. Cancer Vol. 10, pp. 413–418. Pergamon Press 1974. Printed in Great Britain

A Second Comparative Trial of Remission Induction (by Cytosine Arabinoside Given Every 12 Hours, or C.A.R. and Thioguanine, or C.A.R. and Daunorubicine) and Maintenance Therapy (by C.A.R. or Methylgag) in Acute Myeloid Leukaemia

"LEUKAEMIA AND HAEMATOSARCOMA" COOPERATIVE GROUP*
OF THE EUROPEAN ORGANISATION FOR RESEARCH ON THE
TREATMENT OF CANCER (E.O.R.T.C.)

Abstract—*A comparative trial was undertaken to compare cytosine arabinoside (CAR), given every 12 hr, with a combination of CAR and thioguanine (6TG), and with a combination of CAR and daunorubicine (DRB) in the remission induction of acute myeloid leukaemia (AML), and to compare maintenance treatments with CAR and with methylglyoxal-bis guanyl hydrazone (MGGH) on the same patients.*

There was no significant difference in the percentages of remission induction between the three groups of patients (CAR 25%; CAR+6TG 30%; CAR+DRB 31%). The rates of remission were better in the Centres equipped with haematological intensive care units (45·5%).

The duration of the first remissions and of survivals was not influenced significantly by the choice of either the induction or maintenance therapy.

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In a previous randomized trial the group compared the complete remission induction effect in acute myeloid leukaemia (AML) of (a) cytosine arabinoside (CAR) given daily in a four-hour infusion with the following choice as published by Henderson *et al.* [1] in courses of four days of treatment; (b) as a combination of CAR and 6 thioguanine (6TG) [2, 3]; (c) as a combination of CAR and daunorubicine DRB given on the first day of every course of treatment [2, 4].

The results [5] were disappointing, the number of remissions not exceeding 20% although somewhat better results were seen in centres with haematological intensive care units (28% of remissions).

The group decided, therefore, to carry out a second randomized trial using the mode of administration of CAR proposed by Clarkson [3]: the drug is given every 12 hr during 7–10 days for each course of treatment. CAR was, as in the first trial, given either alone, or with 6TG, or with DRB.

Before patients were included in the trial all bone marrow smears were reviewed in a "double blind fashion" by the WHO Reference Center for Leukemias and Hematosarcomas (G. Mathé, I.C.I.G., Hôpital Paul-Brousse, Villejuif), and so were all bone marrow smears showing complete remission (CR). Also the results obtained in the medical departments with haematological reanimation units (platelet and white cell transfusions, aseptic hospitalisation units) [6–8] were compared with those from departments without such a unit.

MATERIAL AND METHODS

1. *The patients and the criteria for diagnosis and evaluation of results*

Two-hundred and eighteen patients with AML were entered into the trial, and of these 179 were available for evaluation. Thirty-nine patients were excluded, seven of these because re-examination of the marrow smears did not support the diagnosis of acute myeloid leukaemia, the other 32 because documentation or follow ups were inadequate.

Patients considered suitable for the trial were entered through registration at WHO Reference Center for Leukemias and Hematosarcomas at the Institut de Cancérologie et d'Immunogénétique (ICIG) in Villejuif (France). Examination of smears and random assignment was completed within 24 hr. Confirmation of inclusion in the study awaited the result of examination and classification of the marrow. Patients were randomized so as to take into account their age and the proportion of leukaemic cells present in the marrow.

The bone marrow smears were classified on the basis of the subdivision of acute leukaemia proposed by Mathé *et al.* [9], the nomenclature of which is that used and illustrated by the WHO Monograph on "Histological typing of the neoplastic diseases of the haematopoietic and lymphoid tissues" [10].

At the completion of therapy with one of the three regimes, the bone marrows were examined again. "Complete remission" was said to be present if the patient was clinically free from disease, if he had a normal peripheral blood with no leukaemia cells present, and if a double blind examination at the WHO Center revealed less than 5% of leukaemic cells in a bone marrow of normal cellularity.

2. *Chemotherapy for remission induction*

The three treatment groups are shown in Table 1. The amount of drugs given in the various courses was constant but in the presence of marrow hypoplasia, the interval between courses was increased. The start of a treatment or a course was postponed in the presence of infection to allow it to be brought under control.

Table 1. Induction treatment schedules

Treatment 1	CAR alone 100 mg/m² IV every 12 hr for 7–10 days. Rest period: 2 or 3 weeks. A total of 3 courses of treatment.
Treatment 2	CAR 100 mg/m² IV every 12 hr for 7–10 days. Rest period: 2 or 3 weeks. A total of 3 courses of treatment. 6TG 80 mg/m² every 12 hr for 7–10 days. First dose given 6 hr before CAR.
Treatment 3	CAR 100 mg/m² IV every 12 hr after DRB for 7–10 days. Rest period: 2 or 3 weeks. A total of 3 courses of treatment. DRB 60 mg/m² IV day 1.

3. *Chemotherapy for remission maintenance*

A comparison of maintenance treatment using methylglyoxal-bis (guanyl-hydrazone)

Table 2. Maintenance treatment schedules

MGGH 200 mg/m² weekly IV or CAR 120 mg/m² weekly SC

(MGGH) or CAR, given as shown in Table 2, was studied in patients who achieved a complete remission. The duration of complete remission and survival were used as the measure of the action. These patients had an examination of the peripheral blood once a week and a bone marrow aspiration when indicated. The marrow examination was also subjected to verification.

4. *Distribution of the patients after randomization*

Forty-eight patients received CAR alone, 70 patients received CAR and 6TG, and 61 patients received CAR and DRB. There were no statistically significant differences between the three groups for remission induction by chemotherapy in regard to sex, age, bleeding symptoms, peripherical blood cell counts, and cytological varieties.

RESULTS

1. *First remissions*

The incidence of complete remissions is shown in Table 3. The mean rate for the group

Table 3. Repartition of remissions according to type of treatment

	Complete remissions	No complete remissions	Total
CAR	12 (25%)	36	48
CAR+6TG	21 (30%)	49	70
CAR+DRB	19 (31%)	42	61
Total	52	127	179

as a whole was 29%. The rates of remissions ranged from 25% for CAR+DRB, to 31% with CAR+DRB. These differences are not statistically significant.

These rates of complete remission are still low but better than those observed in the first trial: 16%, 15% and 21% respectively. The average number of courses of treatment necessary for remission induction was not different from one group to another.

A comparison of the percentages of remissions obtained in our first trial [5] and in this second trial shows a statistically significant improvement (Table 4).

Table 4. Comparison of the repartition of remission in the two trials AML_1 and AML_2

	Complete remissions	No complete remissions	Total
AML_1	30 (18%)	137	167
AML_2	52 (29%)	127	179

Significant $P < 0{\cdot}05$.

Another interesting comparison is between the rates of remission according to availability of "intensive haematological care facilities" such as platelet and white cell transfusions, facilities for isolation, etc.

As in our first trial the remission induction rate was better in the centre with these facilities and reached nearly twice the remission rate of the other centres (Table 5), i.e. 45·5% compared to 25·5%.

Table 5. Comparison of medical centres with haematological reanimation units and other centres

	Complete remission	Non complete remission	Total
Centres with haematological reanimation units	15 (45·5%)	18	33
Centres without these means	37 (25·5%)	109	146
Total	52	127	179

Significant $P < 0{\cdot}05$.

There were no differences in the remission induction rate in respect to age, sex and cytological varieties.

The rate of remission was better, (37%) in the group having an initial number of leucocytes lower than 2×10^4 (Table 6).

The survival of patients having obtained a complete remission (median survival time 16 months) was much better than that of patients not having had a complete remission (median survival time shorter than 2 months) (Fig. 1).

Table 6. Frequence of complete remission in relation to the initial number of leucocytes

Number of leucocytes	Complete remission
$< 2 \cdot 10^4$	37%
$> 2 \cdot 10^4$	16%

Significance $P < 0{\cdot}02$.

2. *Toxicity of induction treatment*

It is difficult in a condition like acute myeloid leukaemia to assess the morbidity or mortality associated with a particular regime. Deaths occurring during the hypoplastic phase resulting from a course of therapy or during the next fifteen days when leucopenia or thrombocytopenia resulted in infection or haemorrhage were considered to result from therapy. There were 9 deaths in the group receiving CAR alone, 6 deaths in the group receiving CAR + 6TG, and 10 deaths in the group receiving CAR + DRB. Most of these deaths occurred during the first month of treatment. The differences are not statistically significant.

3. *Maintenance of remissions*

The actuarial curves of duration of remission for patients in complete remission did not show any difference of statistical significance between the two maintenance treatments (CAR or methyl-glyoxal-bis (guanyl-hydrazone) (MGGH)) (Fig. 2), or the induction treatment (CAR, or CAR + 6TG, or CAR + DRB) (Fig. 3), the median duration of the first remission being between 8 and 10 months.

4. *Survival*

The curves of actuarial survival of patients entering remission were different with different induction treatments (Fig. 4). The survival of patients in remission from CAR alone, or CAR + 6TG (median more than 18 months) seemed better after one year than the survival of patients in remission from CAR + DRB (median survival 13 months). However these differences are not yet statistically significant, few patients having had long enough follow up, but the trend seems clear.

The difference in the actuarial curves of survival was also influenced by the type of maintenance treatment (Fig. 5). Seventy per cent

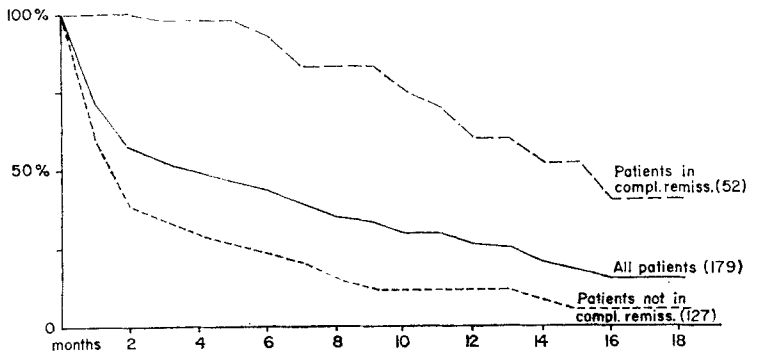


Fig. 1. Actuarial curves of survival: patients having obtained a complete remission; patients not having obtained a complete remission.

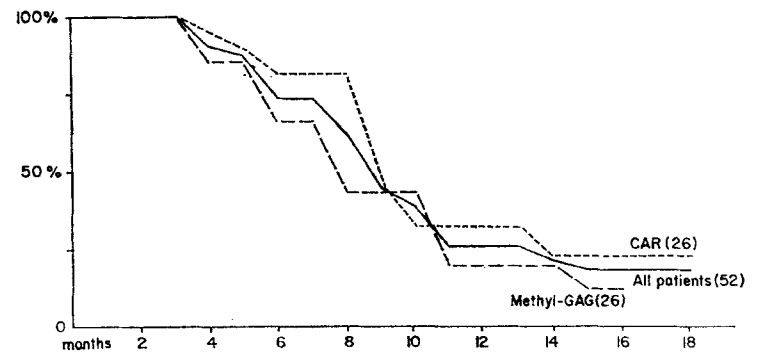


Fig. 2. Actuarial curves of duration of remission for patients in complete remission in relation to maintenance treatment.

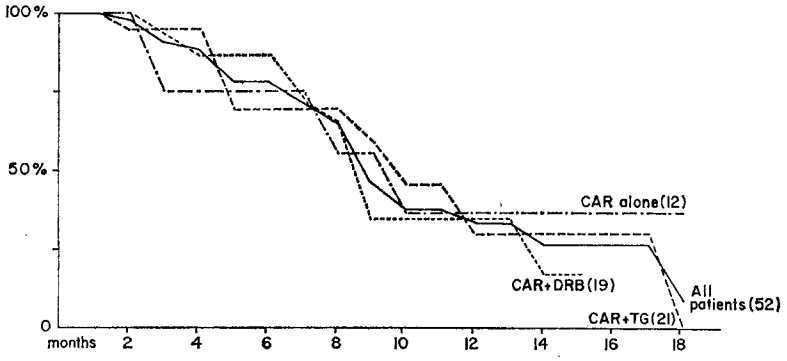


Fig. 3. Actuarial curves of duration of remission for patients in complete remission in relation to induction treatment.

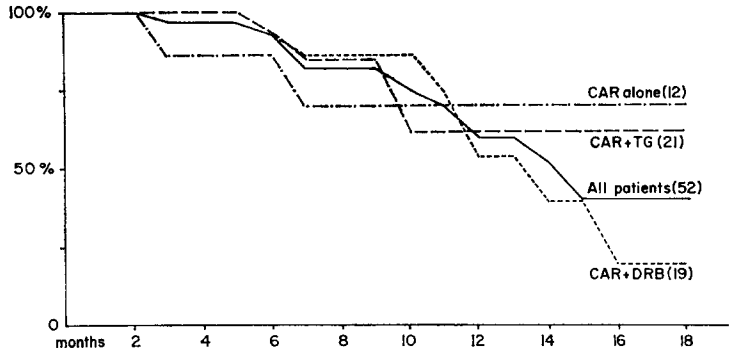


Fig. 4. Acturial curves of survival for patients in complete remission in relation to induction treatment.

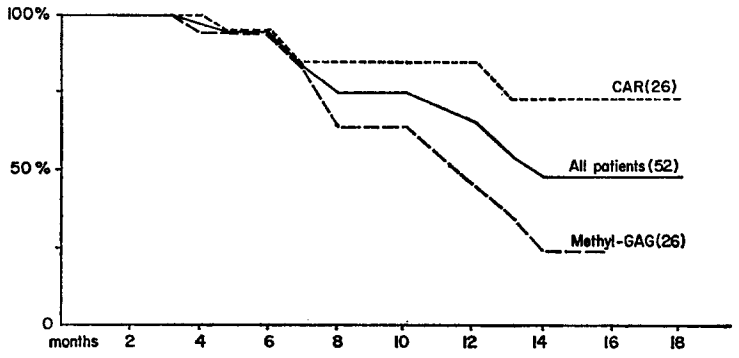


Fig. 5. Actuarial curves of survival for patients in complete remission in relation to maintenance treatment.

survived at 18 months, if the patients received CAR and 25% survived at 18 months if they received MGGH (median survival time 12 months).

As the curves of duration of first remission are not very different, this difference may express a delayed toxicity of DRB and MGGH.

DISCUSSION

In this second trial, as in our first, the percentages of complete remissions did not show statistically significant differences in the different therapeutic class. Nevertheless some points of interest have appeared.

Firstly, the overall rate of remissions was better than in the first trial (29% compared to 18%) and this difference may be related to the time interval for the administration of CAR (every 12 hr), which seemed more efficient than the administration by one 4 hr infusion.

Preliminary data suggest that a 24 hr infusion of CAR may be an even better method of administration.

It appears also that better results were obtained in centres with haematological intensive care units. In these centres 45·5% remissions were obtained, which is comparable to the percentage of remissions published by other groups [3, 4, 11–13]. These results confirm that as far as possible patients with acute leukemia should be treated in highly specialised centres.

Another point of interest is the difference in survival curves with differences in the induction treatment and the maintenance treatment for patients having entered remission. The results are in favour of protocols using CAR, or CAR + 6TG, and they show the long lasting toxic effects of treatments which are very meylotoxic, such as DRB and MGGH.

REFERENCES

1. E. Henderson, A. Serpick, B. Leventhal and P. Henry, Cytosine arabinoside infusions in adult and childhood acute myelocytic leukemia. *Proc. Amer. Assoc. Cancer Res.* **9,** 29 (1968).
2. R. W. Carey, Comparative study of cytosine arabinoside therapy alone and combined with thioguanine or mercaptopurine and daunorubicine in acute myelocytic leukemia. *Proc. Amer. Assoc. Cancer Res.* (Abst. No. 55) **11,** 15 (1970).
3. B. D. Clarkson, M. D. Dowling, T. S. Gee and J. H. Burchenal, In *Treatment of Acute Myeloblastic Leukaemia* Vol. 1 Karger, Basel (1972).
4. D. Crowther, C. J. T. Bateman, C. P. Vartan, J. M. A. Whitehouse, J. S. Malpas, G. Hamilton-Fairley and R. Bodley Scott, Combination chemotherapy using L-asparaginase, daunorubicine and cytosine arabinoside in the treatment of acute myelogenous leukemia. *Brit. Med. J.* **4,** 513 (1970).
5. E.O.R.T.C. Leukaemia and Haematosarcoma Cooperative Group, A comparative trial of remission induction (by cytosine arabinoside, or CAR and thioguanine, or CAR and daunorubicine) and maintenance therapy (by CAR or methylgag) in acute myeloid leukaemia. *Biomedicine, Europ. J. Clin. Biol. Res.* **18,** 192 (1973).
6. G. Mathé and P. Forestier, Un outil moderne de la recherche médicale française, l'Institute de Cancérologie et d'Immunogénétique. Intrications de la recherche expérimentale et clinique. Conditionnement hyposeptique des animaux et des malades. *T ch. Hosp.* **20,** 47 (1965).
7. L. Schwarzenberg, A. Caïtan, M. Schneider, J. R. Schlumberger, J. L. Amiel and G. Mathé, La réanimation hématologique. I. Corrections des désordes graves de la lignée érythrocytaire et des désordres de la coagulation. *Presse Med.* **74,** 969 (1966).
8. L. Schwarzenberg, A. Caïtan, M. Schneider, J. R. Schlumberger, J. L. Amiel and G. Mathé, La réanimation hématologique. II. Correction des désordres graves des leucocytes et des immunoglobulines. La greffe de moelle osseuse. *Presse Med.* **74,** 1061 (1966).
9. G. Mathé, P. Pouillart, M. Steresgu, J. L. Amiel, L. Schwarzenberg, M. Schneider, M. Hayat, F. De Vassal, C. Jasmin and M. Lafleur, Subdivision of classical varieties of acute leukaemia. Correlation with prognosis and cure expectancy. *Europ. J. Clin. Biol. Res.* **16,** 554 (1971).
10. G. Mathé and H. Rappaport, Histocytological typing of the neoplastic disease of the haematopoietic and lymphoid tissues. W. H. O. Geneva (1973).
11. D. Crowther, R. Powles, C. J. T. Bateman, M. E. J. Beard, C. L. Gauci, P. F. M. Wrigley, J. S. Malpas and G. Hamilton-Fairley, Management of adult myelogenous leukaemia. *Brit. Med. J.* **1,** 131 (1973).
12. T. S. G. Gee, K. P. Yu and B. D. Clarkson, Treatment of adult acute leukaemia with arabinosyl cytosine and thioguanine. *Cancer* **23,** 1019 (1969).
13. D. S. Rosenthal and W. C. Moloney, The treatment of acute granulocytic leukaemia in adults. *New Engl. J. Med.* **286,** 1176 (1972).

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Oncorna-Viral Information in Human Melanoma

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Abstract—*Human malignant melanomas were investigated for the presence of oncorna-viral information by chemical and electron microscopical methods. In all primary and metastatic tumors a RNA-instructed DNA polymerase was detected. This enzymic activity exhibits features characteristic for that of oncogenic RNA viruses. A high molecular weight (70S) RNA species, a further diagnostic feature for oncorna virions, was found in the subcellular fraction with the highest RNA-instructed DNA polymerase activity. In addition, by electron microscopy particles of a morphology similar to C-type virions were found in this subcellular fraction.*

INTRODUCTION

EXTENSIVE studies on animal melanomas (especially that of the hamster), which serve as model for human malignant melanoma have shown the occurrence of virus particles in the tumors [1,2]. A high molecular weight (70S) RNA species as well as a RNA-directed DNA polymerase, two diagnostic features for oncorna (oncogenic RNA) viruses have been associated with these particles [3–5]. Cell-free transmission experiments end to the conclusion that hamster melanomas are caused by an RNA virus [6, 7].

Based on these findings the working hypothesis was proposed that human melanoma may also have a viral etiology. In preliminary experiments we have reported the occurrence of virus-like particles [8] as well as reverse transcriptase activity [9] in metastases of human melanoma. This paper describes more detailed studies of oncorna-viral information in human melanoma.

MATERIALS AND METHODS

Tissue preparation

The tumors used in our studies were primary melanoma as well as metastases to lymph nodes and brain. They were processed immediately after surgical removal. The material was dissected, suspended in 0·25 M sucrose in TNE buffer (10 mM Tris–HCl, 150 mM NaCl and 2 mM EDTA), pH 8·3, and homogenized with an Ultra-Turrax at low speed. The tumor cells were then broken up in a glass–teflon homogenizer, and nuclei and cell debris were removed by centrifugation for 5 min at 3000 *g*. Crude mitochondria were sedimented by centrifugation for 10 min at 10,000 *g*. The mitochondrial supernatant was layered on a cushion of 10 ml 20% glycerol in TNE, pH 8·3, and centrifuged for 1 hr at 27,000 rev/min in a Beckman SW 40 rotor. The sediment of this centrifugation was denoted MC-P100. When this sediment contained more than 10 mg protein, it was further separated on a linear sucrose gradient ranging from 25 to 50%. Velocity sedimentation was performed for 5 hr at 40,000 rev/min in a Beckman SW40 rotor. The tubes of the gradient were eluted and the optical density recorded simultaneously at 280 nm. Fractions of 0·4 ml were collected and pooled for each protein peak, and the protein was pelleted by centrifugation for 1 hr at 100,000 *g*.

Measurement of total DNA polymerase activity

DNA polymerase was assayed by determining the incorporation of a radioactive-labelled

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precursor into an acid-insoluble state [10]. Protein samples (usually 0·150 mg) were suspended in TNM buffer (10 mM Tris–HCl, 150 mM NaCl and 5 mM $MgCl_2$), pH 8·3, containing 0·1% Triton X-100 and 25 mM dithiothreitol, and incubated for 15 min at 4°C. The reaction was started by addition of the protein to the reaction mixture preincubated at 37°C. The incubation mixture of 0·26 ml contained in μmoles: 2·08 Tris–HCl, pH 8·3, 0·26 $MgCl_2$, 0·325 NaCl, 0·052 of each dATP, dCTP and dGTP and 0·715 nmoles of ^{3}H-TTP. Unless otherwise specified the reactions were carried out at 37°C for 30 min and stopped by addition of 10% perchloric acid containing 50 mM sodium pyrophosphate. The acid precipitable radioactivity was reprecipitated in ethanol. After dissolution in concentrated formic acid the samples were counted in a Packard liquid scintillation counter using tert.butyl-PBD scintillation fluid.

Simultaneous detection test of 70S RNA and reverse transcriptase

The simultaneous detection test of reverse transcriptase and high molecular weight RNA was performed according to Schlom and Spiegelman [11]. A standard reverse transcriptase reaction was performed for 15 min at 37°C. The reaction was stopped by the addition of NaCl and SDS (sodium–dodecylsulfate) to final concentrations of 0·2 M and 1%, respectively. After addition of an equal volume of a mixture of phenol and *m*-cresol (7:1, pH 7·6) containing 8-hydroxychinoline (3·7 *g* per 100 ml), the final mixture was shaken at 25°C for 5 min and centrifuged at 5000 *g* for 5 min. The aqueous phase was then layered over a linear glycerol gradient (10–30%) and centrifuged at 40,000 rev/min for 5 hr at 4°C in a Beckman SW 40 rotor. The gradient was eluted with 40% glycerol from the bottom of the tube. Simultaneously the optical density at 260 nm was recorded in a flow-through cuvette of a Gilford 2400 S spectrophotometer. Fractions of 0·4 ml were collected and the radioactivity was measured after addition of 50 μg unlabelled RNA and precipitation of the nucleic acids by perchloric acid.

Electron microscopy

The subcellular fractions were sedimented for 1 hr at 100,000 *g* and the pellets were fixed in 1% OsO_4 in 0·1 M cacodylate buffer, pH 7·2, for 1 hr. The samples were then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut on a LKB Ultrotome III with a diamond knife, mounted on copper grids covered with collodium and carbon films, stained with uranyl acetate [12] and lead citrate [13] and examined in a Siemens Elmiskop IA or 101 at 80 kV.

RESULTS

RNA-instructed DNA polymerase activity was detected in all primary melanomas (9 cases) as well as in all metastases to regional lymph nodes (5 cases) as shown in Table 1. A brain metastasis contained similar activity. The specific activity of the DNA polymerase measurable in the microsomal fraction MG-P100 from human melanoma was comparable to that of the hamster melanoma A Mel 3, which is known to contain a RNA tumor virus [14].

Table 1. RNA-directed DNA polymerase in a subcellular fraction (MC-P100) isolated from human melanoma

	Specific activity (*p*-moles ^{3}H-TMP incorporated per min and mg protein)	% Inhibition by Ethidium-bromide	% Inhibition by Actino-mycin
Primary human melanoma (9 cases)	$1{\cdot}37 \times 10^{-2} \pm 0{\cdot}22$	78	30
Lymph-node metastases (5 cases)	$1{\cdot}32 \times 10^{-2} \pm 0{\cdot}28$	82	35
Brain metastasis (1 case)	$1{\cdot}54 \times 10^{-2} \pm 0{\cdot}00$	85	28
Human skin	$0{\cdot}14 \times 10^{-2} \pm 0{\cdot}01$	20	85
Hamster melanoma A Mel 3	$2{\cdot}10 \times 10^{-2} \pm 0{\cdot}15$	90	30

Actinomycin D, an inhibitor of DNA-dependent nucleic acid polymerases [15] reduced the activity to about 30%, whereas the phenanthridine dye ethidiumbromide inhibited the reverse transcriptase much more effectively.

In the corresponding subcellular fraction MG-P100 isolated from normal human skin very little DNA polymerase activity could be detected (Table 1, 4th row). In contrast to the tumor enzyme, this activity was very sensitive to actinomycin D but little sensitive to ethidiumbromide.

An appreciable quantity of MG-P100 was obtained from the brain metastasis. This material was further separated by density gradient centrifugation into 4 protein fractions. The fourth protein peak sedimenting at a density of 1·19 g/cm^3 exhibited the highest reverse transcriptase activity. The specific activity of this fraction was about twice that of MG-P100, and allowed the determination of some of the characteristic features of the DNA polymerase activity. They are summarized in Table 2. The enzymic activity was very sensitive to preincubation with RNase, indicating that its action is RNA dependent. The stimulation by the synthetic polynucleotide poly rA:oligo dT_{10} is similar to that of reverse transcriptase isolated from various RNA tumor viruses. This argues for reverse transcriptase being present in human melanoma.

Table 2. RNA-directed DNA polymerase of the subcellular fraction M-PG100/peak 4 isolated from a brain metastasis of human melanoma

Conditions	Specific activity (*p*-moles ^{3}H-TMP incorporated per min and mg protein)	% Control
MG-P100/peak 4	$3{\cdot}03 \times 10^{-2}$	100
$-Mg^{2+}$		15
−dCTP, dGTP, dATP		5
preincubation with RNase		12
+polydT:rA		550
+23S RNA (*E. coli*)		100
+Actinomycin D (50 μg/ml)		72
+Ethidiumbromide (50 μg/ml)		15

A second diagnostic feature for oncogenic RNA viruses [16], a single stranded high molecular weight (70S) RNA, could be identified simultaneously with the reverse transcriptase activity. The result of the simultaneous detection test is given in Fig. 1. It shows the labelling pattern in the glycerol gradient of the nucleic acid extracted from MG-P100 after a standard DNA polymerase reaction. Two nucleic acid fractions, one of low and one of high molecular weight, have been obtained.

Most of the radioactivity incorporated by the DNA polymerase was found in the low molecular weight region. A significant label, however, is associated with the high molecular weight region. This radioactivity can be destroyed by incubation with RNase prior to gradient centrifugation. The elution profile and its labelling pattern indicated the occurrence of a single stranded high molecular weight RNA in human melanoma serving as a template for the RNA-directed DNA polymerase.

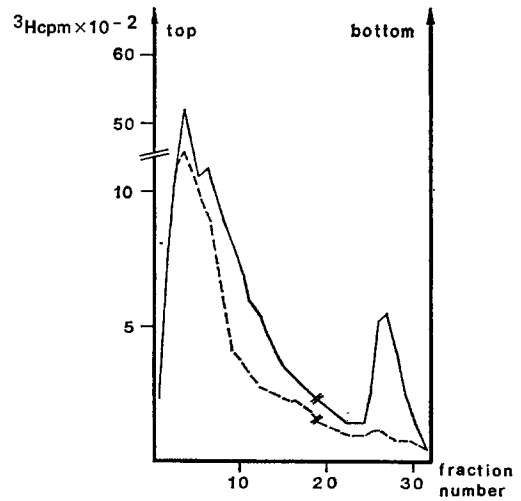


Fig. 1. Simultaneous detection of a high molecular weight RNA and a RNA-directed DNA polymerase in a subcellular fraction of human melanoma.

Full line: simultaneous detection test without preincubation of the extracted nucleic acid with RNase.

Broken line: simultaneous detection test with preincubation with RNase.

In addition to the biochemical evidence for the presence of viral information in human melanoma ultrastructural studies have been performed with the aim to detect particles which may allow a correlation with the viral RNA-directed DNA polymerase.

In electron micrographs of the subcellular fraction MG-P100 of different melanomas, one primary tumor (Fig. 2) and one metastasis (Fig. 3), round or slightly ovoid particles could be detected which are characterized by an electron dense core. They are limited by a triple-layered membrane and their average size amounts to 120 nm. Their morphological appearance is similar to that of RNA tumor viruses of the C-type [17].

DISCUSSION

The search for a human melanoma virus was prompted by the existence of such an agent in mouse and hamster tumors [18, 6] and from the immunological findings of a tumor-specific antigen in human melanoma [19, 20]. In

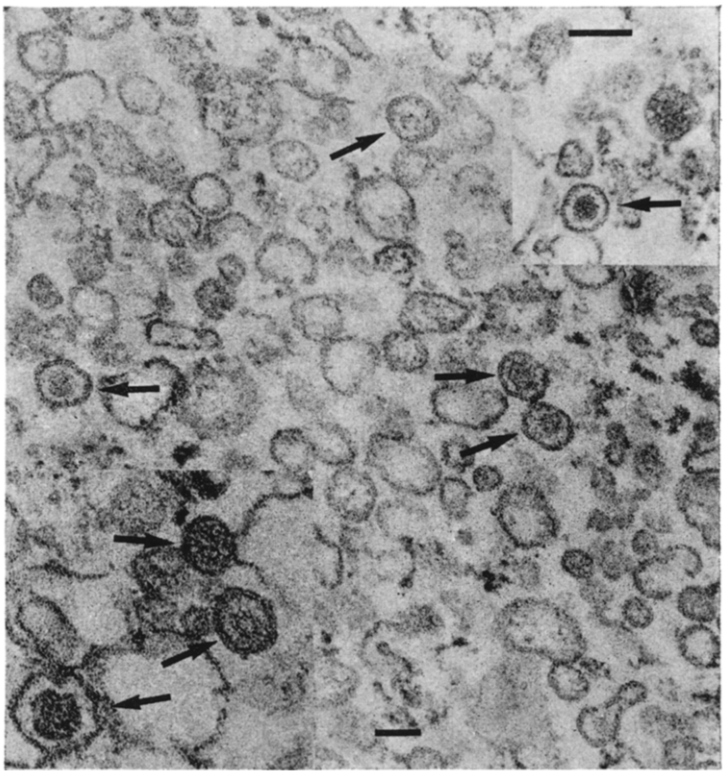


Fig. 2. Subcellular fraction (MG-P100) of primary human melanoma. Virus particles (arrows) contain an electron dense core and are bounded by a triple-layered membrane. Identical particles in the insets are from other sections of the same fraction. 100·000× (*Insets:* 140·000×) *Bars indicate* 0·1 *μm.*

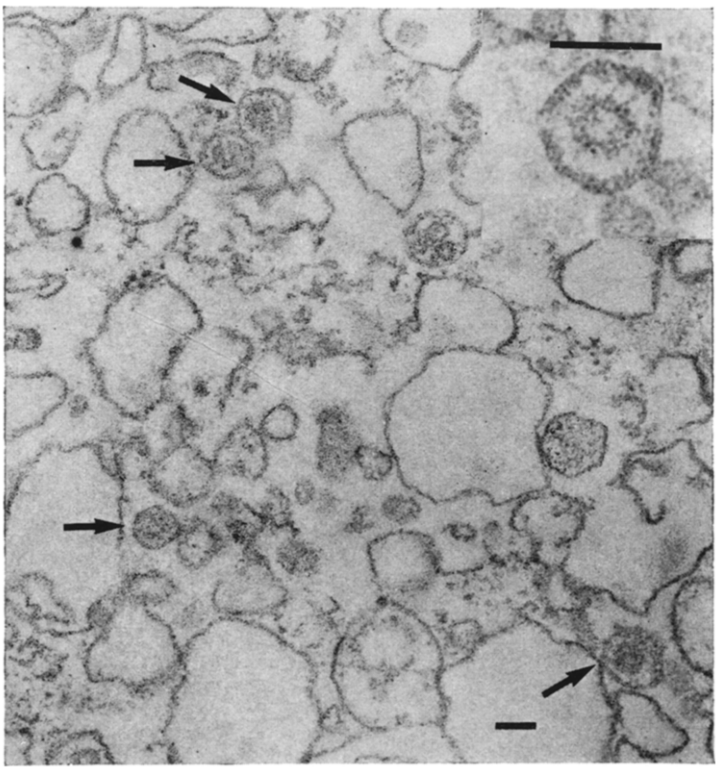


Fig. 3. Subcellular fraction (MG-P100) of brain metastasis of human melanoma. Virus particles (arrows) are morphologically identical with those seen in the primary tumor. Inset shows a particle with a core more translucent in the center and surrounded by a membrane appearing triple-layered at right. 86·000× (*Inset:* 248·000×). *Bars indicate* 0·1 *μm.*

(to face p. 422)

animal melanomas virus particles have been detected by electron microscopy [21]. Furthermore, the morphological findings could be correlated with two biochemical features for oncongenic RNA viruses, a single stranded high molecular weight (70S) RNA and RNA–instructed DNA polymerase [22] which were identified in hamster melanomas [5]. The presumed viral etiology of this tumor has been verified by a successful cell-free transmission [6, 7].

A definitive proof for the viral etiology of human cancer cannot be obtained on the basis of cell-free transmission. However, this limitation can partially be overcome if the postulates of an infectious agent as a cause of cancer are reduced to the following question: do malignant tumors contain oncorna-viral information not found in corresponding normal tissue?

Electron microscopic observations have indicated the occurrence of virus particles in some human malignancies such as breast cancer [23] and leukemia [24].

Biochemical findings in favour of the viral etiology of human cancer are the demonstration of viral-specific RNA in human breast carcinomas [25], leukemias [26], sarcomas [27] and lymphomas [28] by molecular hybridization, the detection of reverse transcriptase employing a 70S RNA template in human leukemic cells [22] and breast cancer [29] and the presence of 70S RNA and reverse transcriptase in certain samples of human milk [30]. The following criteria may therefore be used in the search for a human tumor virus:

(i) presence of a RNA-instructed DNA polymerase,
(ii) presence of high molecular weight (70S) RNA,
(iii) virus-specific nucleic acid sequences,
(iv) antigens related to known RNA tumor viruses.

As shown in this communication, evidence for the first two criteria has been obtained for human melanoma, further established by detection of virus-specific nucleic acid sequences by molecular hybridization techniques [31].

Since a RNA-dependent DNA polymerase activity has also been detected in normal cells [32, 33] we have looked for reverse transcriptase activity in normal human skin and found only very little of a somewhat different polymerase activity. It could not be stimulated by the synthetic polynucleotide poly rA:oligo dT_{10}. In contrast, the melanoma enzyme was stimulable by poly rA:oligo dT_{10} and employed a high molecular weight RNA template. In addition, our biochemical findings are supported by the electron microscopical observations. We found particles similar to, but not identical with, C-type viruses, but their number is small in comparison to that found in the corresponding fractions of the hamster melanoma [7]. A much larger amount of these particles, which may not be obtainable from biopsy material, is needed for their characterization by chemical and immunological means.

To overcome this difficulty studies are in progress to induce a virus production in melanoma cells propagated *in vitro*.

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REFERENCES

1. B.-R. Balda, H.H. Wolff, G.D. Birkmayer and O. Braun-Falco, Virus-like particles in the hamster melanoma A Mel 3 of Fortner. *Naturwissenschaften* **57,** 548 (1970).
2. B.-R. Balda, Zur Molekularbiologie des Hamstermelanoms A Mel 3 einem möglichen Modell für das menschliche maligne Melanom. Habilitationsschrift, Universität München (1971).
3. E. Albu and K. V. Holmes, Isolation and characterization of the virus-like particles of BHK 21 cell. *J. Cell Biol.* **55,** 1 a (1972).
4. G. D. Birkmayer, F. Miller and B.-R. Balda, Inhibition of high molecular weight RNA synthesis in a hamster melanoma by ethidium bromide *in vivo*. *Hoppe-Seyler's Z. phys. Chem.* **353,** 1749 (1972).
5. B.-R. Balda and G. D. Birkmayer, Particle—associated RNA—directed DNA—Polymerase and 70 S RNA in the hamster melanoma A Mel 3. *Arch. Derm. Res.* **248,** 229 (1973).
6. W. L. Epstein, K. Fukuyama, M. Benn, A. S. Keston and R. B. Brandt, Transmission of a pigmented melanoma in golden hamster by a cell—free ultrafiltrats. *Nature (Lond.)* **219,** 979 (1968).

7. G. D. Birkmayer, B.-R. Balda and F. Miller. The inhibitory effect of proflavine and ethidiumbromide on the cell-free transmission and the growth of the hamster melanoma A Mel 3. *Europ. J. Cancer* **9,** 859–864.
8. G. D. Birkmayer, B.-R. Balda, F. Miller and O. Braun-Falco. Virus-like particles in metastases of human malignant melanoma. *Naturwissenschaften* **59,** 369 (1972).
9. B.-R. Balda and G. D. Birkmayer, Further evidence for the viral etiology of human melanoma. *Naturwissenschaften* **60,** 304 (1973).
10. A. C. Garapin, J. P. McDonnell, W. Levinson, N. Quintrell, L. Fanshier and J. M. Bishop, Deoxyribonucleic acid polymerase associated with rous sarcoma virus and avian myeloblastosis virus: properties of the enzyme and its product. *J. Virol.* **6,** 589 (1970).
11. J. Schlom and S. Spiegelman. Simultaneous detection of reverse transcriptase and high molecular weight RNA unique to oncogenic RNA viruses. *Science* **174,** 840 (1971).
12. J. M. Frasca and V. R. Parks, A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* **25,** 157 (1965).
13. J. H. Venable and R. Coggeshall, A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25,** 407 (1965).
14. G. D. Birkmayer, Zell- und molekularbiologische Indizien für die Virusätiologie des Melanoms. Habilitationsschrift, Universität München (1973).
15. J. Hurwitz, J. J. Furth, M. Malama and M. Alexander, The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by actinomycin D and proflavine. *Proc. nat. Acad. Sci. U.S.A.* **48,** 1222 (1962).
16. S. C. Gulati, R. Axel and S. Spiegelman, Detection of RNA-instructed DNA polymerase and high molecular weight RNA in malignant tissue. *Proc. nat. Acad. Sci. U.S.A.* **69,** 2020 (1972).
17. W. Bernhard, The detection and study of tumor viruses with the electron microscope. *Cancer Res.* **20,** 712 (1960).
18. F. Hu and P. F. Lesley, The isolation and cytology of two pigment cell strains from mouse melanoma. *Cancer Res.* **24,** 1634 (1964).
19. S. Carrel and C. Theilkaes, Evidence for a tumour-associated antigen in human malignant melanoma. *Nature (Lond.)* **242,** 609 (1973).
20. G. H. Heppner, L. Stolbach, M. Byrne, F. J. Cummings, E. McDonough and P. Calabresi. Cell-mediated and serum blocking reactivity to tumor antigens in patients with malignant melanoma. *Int. J. Cancer* **11,** 245 (1973).
21. R. Ito and Y. Mishima, Particles in the cisternae of the endoplasmic reticulum of Fortner's amelanotic and melanotic malignant melanomas. *J. Invest. Derm.* **48,** 268 (1967).
22. W. Baxt, R. Hehlman and S. Spiegelman, Human leukemic cells contain reverse transcriptase associated with a high molecular weight virus-related RNA. *Nature, New Biol.* **240,** 72 (1972).
23. G. Seman, H. S. Gallager, J. M. Lukeman and L. Dmochowski, Studies on the presence of particles resembling RNA virus particles in human breast tumors, pleural effusions, their tissue cultures and milk. *Cancer* **28,** 1431 (1971).
24. L. Dmochowski, T. Yumoto, C. E. Grey, R. Hales, P. L. Langford, H. G. Taylor, E. J. Freireigh, C. C. Shullenberger and J. A. Shively, Electron microscopic studies of human leukemia and lymphoma. *Cancer* **20,** 760 (1967).
25. S. Spiegelman, R. Axel and J. Schlom. Virus-related RNA in human and mouse mammary tumors. *J. nat. Cancer Inst.* **48,** 1205 (1972).
26. W. G. Baxt and S. Spiegelman, Nuclear DNA sequences present in human leukemic cells and absent in normal leukocytes. *Proc. nat. Acad. Sci. U.S.A.* **69,** 3737 (1972).
27. S. Spiegelman, R. Axel, W. Baxt, S. C. Gulati, R. Hehlmann, D. Kufe and J. Schlom, Human cancer and the RNA tumor viruses. In *RNA Viruses, Structure and Function-Ribosomes, Structure, Function and Biogenesis.* Proc. 8th Meet. FEBS Amsterdam, **27,** 73 (1972).

28. R. Hehlmann, D. Kufe and S. Spiegelman, Viral-related RNA in Hodgkins' disease and other human lymphomas. *Proc. nat. Acad. Sci. U.S.A.* **69,** 1727 (1972).
29. R. Axel, S. C. Gulati and S. Spiegelman, Particles containing RNA-instructed DNA polymerase and virus related RNA in human breast cancers. *Proc. nat. Acad. Sci. U.S.A.* **69,** 3133 (1972).
30. J. Schlom, S. Spiegelman and D. Moore, RNA-dependent DNA polymerase activity in virus-like particles isolated from human milk. *Nature (Lond.)* **231,** 97 (1971).
31. B.-R. Balda, R. Hehlmann, J.-R. Cho, S. Spiegelman. (In preparation).
32. J. Coffin and H. M. Temin, Ribonuclease-sensitive deoxyribonucleic acid polymerase activity in uninfected rat cells and rat cells infected with Rous sarcoma virus. *J. Virol.* **8,** 630 (1971).
33. D. C. Ward, K. C. Humphryes and I. B. Weinstein, Synthetic RNA-dependent DNA polymerase activity in normal rat liver and hepatomas. *Nature (Lond.)* **237,** 499 (1972).

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Dose-Related Synergism of Cytosine Arabinoside and Methotrexate Against Murine Leukemia L1210*

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Abstract—*When administered in rapid succession, cytosine arabinoside and methotrexate acted synergistically in prolonging the lives of* BDF_1 *mice inoculated with L*1210 *ascites tumor cells. The magnitude of this synergism increased as the dosage of either or both drugs was increased. At maximum dosage* (12 *mg methotrexate per kg plus* 1200 *mg of cytosine arabinoside per kg*), *the drug combination completely eradicated tumor cells in* 11 *of* 40 *mice and extended the mean survival of the remaining* 29 *animals by* 23·64 *days* (332%) *beyond control survival. In contrast, neither drug given singly on the same schedule cured leukemic mice nor extended their mean survival beyond controls by more than* 10·18 *days* (143%). *Statistically significant contributions to survival were made by three experimental variables:* (*i*) *methotrexate dosage,* (*ii*) *cytosine arabinoside dosage, and* (*iii*) *dosage interaction between the two drugs.*

INTRODUCTION

COMBINATIONS of drugs are frequently more effective than single drugs in the treatment of neoplastic diseases [1, 2]. Agents with similar modes of action are usually not combined, although a compelling rationale for this practice has not been established. There are, however, a number of observations in favor of combining two DNA inhibitors, cytosine arabinoside (Ara-C) and methotrexate (MTX). In preclinical studies, Ara-C was active against leukemic cells resistant to MTX [3–5] and, in clinical trials, was active against acute leukemias that were resistant to other agents [6–10]. Moreover, systemically administered Ara-C retarded the proliferation of L1210 meningeal leukemia that arose during treatment with MTX [11]. Biochemically, the two drugs have different sites of action in the synthetic pathway of DNA [12, 13] and, on this basis, could be expected to exert complementary action against neoplasias for which they are independently effective.

On the other hand, in preclinical tests, combinations of Ara-C and MTX produced variable effects ranging from antagonism to synergism. When administered daily to mice with L1210 leukemia, combinations of low dosages of Ara-C and MTX did not have significantly greater activity against leukemic cells than did Ara-C alone, as judged by mouse survival time [14]. With the spleen colony assay, a synergistic effect on L1210 cells

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was noted when the two drugs were combined [15]. More recent reports have indicated that combinations of these drugs are antagonistic *in vitro* [16, 17] and *in vivo* [18].

We have re-examined the effects of various dosages of Ara-C and MTX, given individually or in combination, on the survival of mice inoculated with L1210 ascites tumor cells. Our data indicate a strong dose-related synergism when these two inhibitors of DNA synthesis are administered together.

MATERIAL AND METHODS

Female BDF_1 mice, weighing approximately 18 g each, were maintained under conditions of uniform caging, 75°F temperature, 55% relative humidity, food and water *ad libitum*, and 12-hr photoperiods with the light phase commencing at 06.00 a.m. The mice were inoculated intraperitoneally with one million L1210 ascites tumor cells and treated with Ara-C and/or MTX. Cytosine arabinoside was obtained from the Upjohn Co., and MTX from the National Cancer Institute. The drugs were administered individually by the intraperitoneal route as a volume of 0·01 ml/g of body weight. For multiple-drug therapy the two agents were injected in rapid succession. Control mice received injections of physiological saline.

Treatment schedules considered optimum for MTX and Ara–C given singly were not assumed to be optimum for a combination of the drugs. Treatment was initiated on the day after tumor inoculation (day 1) and repeated on the fourth and seventh days. This schedule approximated a clinical treatment schedule, permitted cumulative drug toxicity, and limited treatment to a time period equivalent to the mean life-span of control mice.

Drug effectiveness was evaluated by comparing the post-inoculation life-spans of treated and control mice. Deaths were attributed to tumor on the basis of specific findings at necropsy. When mice without evidence of tumor or organomegaly died earlier than animals treated with lower dosages of drug, before controls, or two standard deviations earlier than treatment mates, their deaths were attributed to acute drug intoxication. Mice were considered cured if they had no evidence of disease on day 60 after inoculation (i.e. 60–day survivors).

The individual drugs, administered at three levels of dosage, provided three treatment groups per drug. Two-drug experiments were of factorial design with three dosage levels for each drug; this provided nine additional treatment groups. Estimates of central value and dispersion were calculated for each treatment group [19, 20]. Results from two-drug studies were partitioned into their variance components, and an analysis of variance [19–21] was performed to evaluate contributions by single drugs and by drug interaction to variation in survival time. Statistical infer-

Table 1. *Response of leukemic mice to MTX and Ara-C, administered singly or in combination*

Treatment (mg/kg) MTX	Ara-C	No. of Mice	No. of deaths due to tumor	Mean survival ± 1 S.D. (Days after inoculation*)	Cures (60-Day survivors)
0	0	375	375	7·12± 1·16	0
1·3	0	15	15	8·50± 1·60	0
4·0	0	15	15	10·70± 1·40	0
12·0	0	20	20	14·40± 3·71	0
0	432	30	30	12·70± 2·07	0
0	720	30	30	14·10± 2·80	0
0	1200	35	35	17·30± 3·51	0
1·3	432	20	20	15·85± 2·03	0
1·3	720	20	20	16·95± 2·72	0
1·3	1200	20	19	20·11± 2·87	0
4·0	432	20	19	18·32± 2·58	1
4·0	720	20	18	21·67± 2·83	1
4·0	1200	15	14	22·21± 3·91	1
12·0	432	20	17	24·29± 4·81	1
12·0	720	30	23	26·35± 4·44	7
12·0	1200	40	29	30·76± 10·60	11

Four deaths were attributed to drug toxicity; these were nonspecific in origin and did not appear related to drug dosage.

* Mice were inoculated with one million L1210 ascites tumor cells 24 hr before treatment.

Table 2. Analysis of survival variation after treatment with MTX and Ara-C

Source of variation	Degree of freedom	Sum of squares	Mean square	F	Critical value to be exceeded by F for significance at the indicated level of probability	
					$P<0{\cdot}05$	$P<0{\cdot}01$
Total	178	9094	—	—	—	—
Among treatment	8	4326	540·75	19·28	2·02	2·66
Ara-C	2	857	428·50	15·28	3·07	4·79
MTX	2	3122	1561·00	55·65	3·07	4·79
MTX times Ara-C	4	347	86·75	3·09	2·45	3·48
Within treatment	170	4768	28·05	—	—	—

As indicated by the F test, MTX, Ara-C and the interaction between the two drugs each made a significant contribution to variation in survival.

ence was determined by the F-test [19–21]. Linear regression equations [20–21] were calculated to aid the presentation of data.

RESULTS AND DISCUSSION

Presented in Table 1 are the survival times of 350 BDF_1 mice inoculated with L1210 cells and treated with cytosine arabinoside and/or methotrexate on post-inoculation days 1, 4 and 7. Compared to the mean control value, 7·12 days, survival times of treated mice were extended 1·38–7·28 days [19–102%) by MTX and 5·58–10·18 days (78–143%) by Ara-C. The effectiveness of each drug increased with increasing dosage, but neither drug produced cures. No deaths were attributed to drug toxicity.

When administered together in nine combinations of dosage, MTX and Ara-C exerted complementary action against L1210 cells. Each combination produced a synergistic increase in mouse survival; i.e. each increase was greater than the sum of responses to equivalent dosages of the individual drugs. However, substantial variation in survival time was noted among the 183 mice that died from tumor. An analysis of variance (see Table 2) indicated that a significant portion of this variation ($P<0{\cdot}01$) was due to differences in treatment. Among the nine treatment groups, mean survival ranged from 15·85 to 30·76 days, reflecting increases of 8·73–23·64 days (123–332%) over control survival. Statistically significant contributions to survival were assigned to three variables: (i) MTX dosage $P<0{\cdot}01$), (ii) Ara-C dosage ($P<0{\cdot}01$), and (iii) dosage interaction between the two drugs ($P<0{\cdot}05$). For mice that died from tumor, the influence of MTX and Ara-C on survival is expressed by the regression equation $y = 13{\cdot}34 + 0{\cdot}62x + 0{\cdot}0045x' + 0{\cdot}0003xx'$. Methotrexate and Ara-C are represented respectively by x and x', and y represents survival time in days. As expressed by this equation, each mg/kg increase in MTX increased survival by 0·62 days regardless of the accompanying dosage of Ara-C. Similarly, each 100 mg/kg increase in Ara-C increased survival by 0·45 days regardless of the accompanying dosage of MTX.

In addition to these effects, there were increases in survival that could not be assigned mathematically to either drug alone, but could be related to an interaction between the two. Survival was increased an additional 0·0003 by each mg/kg increase in the product of MTX and Ara-C dosages. Since products increase disproportionately to sums, the influence of dosage interaction increased in importance as drug dosages were increased. At lowest dosage (1·3 mg MTX/kg plus 432 mg Ara-C/kg), this factor contributed only 0·18 days to survival, whereas at maximum dosage (12 mg MTX/kg plus 1200 mg Ara-C/kg) it extended survival by 4·32 days.

Tumor cells were completely eradicated in 22 mice receiving combinations of the two drugs. Sixty-day survivors were noted at each level of Ara-C dosage, but at only the intermediate and high dosages of MTX. At maximum dosages of both drugs, tumor was eradicated in 11 of 40 mice. If cured mice are included as 60-day survivors in the computation of mean survival time, maximum survival becomes 38·80 days, an increase of 445% over control survival. In this situation, the influence of combinations of MTX and Ara-C on survival is expressed by the regression equation $y = 14{\cdot}10 + 0{\cdot}61x + 0{\cdot}003' + 0{\cdot}001xx'$.

The most striking result of including cured

mice in the computation of survival is increased emphasis on the dosage interaction between MTX and Ara-C. That is, each mg/kg increase in the product of MTX and Ara-C dosage produced a 0·001-day increase in survival. At lowest dosages, drug interaction contributed 0·56 days to survival, while at maximum dosage it extended survival by 14·40 days.

From these studies, the fundamental qualitative relationship between MTX and Ara-C appears as a dose-related synergism in which the net quantitative effectiveness of each drug is partially dependent upon the dosage of the other drug. The data presented indicate that increased oncolytic effectiveness accompanied increased dosage of either or both drugs. Other investigators, using different experimental models or lower drug dosages on different schedules of administration, have found combinations of methotrexate and cytosine arabinoside less effective [14] and even antagonistic [16–18].

REFERENCES

1. E. S. Henderson and R. J. Samaha, Evidence that drugs in multiple combinations have materially advanced the treatment of human malignancies. *Cancer Res.* **29,** 2272 (1969).
2. A. Goldin, J. S. Sandberg, E. S. Henderson, J. W. Newman, E. Frei, III and J. F. Holland, The chemotherapy of human and animal acute leukemia. *Cancer Chemother. Rep.* **55,** 309 (1971).
3. J. S. Evans, E. A. Musser, L. Bosturck and G. D. Mengel, The effect of 1-β-D-arabinofuranosylcytosine hydrochloride on murine neoplasms. *Cancer Res.* **24,** 1285 (1964).
4. I. Wodinsky, Activity of cytosine arabinoside (NSC-63878) in a spectrum of rodent tumors. *Cancer Chemother. Rep.* **47,** 65 (1965).
5. D. Tyrer, I. Kline, J. M. Venditti and A Goldin, Effectiveness of combination therapy with cytosine arabinoside (Ara-C) and methotrexate (MTX) in mice inoculated with a mixture of Ara-C and MTX-resistant leukemia L1210 cells. *Proc. Amer. Ass. Cancer Res.* **8,** 68 (1967). (Abstract)
6. E. S. Henderson and P. J. Burke, Clinical experience with cytosine arabinoside. *Proc. Amer. Ass. Cancer Res.* **6,** 26 (1965). (Abstract)
7. R. Papac, W. A. Creasy, P. Calabresi and A. D. Welch, Clinical and pharmacological studies with 1-β-arabinofuranosylcytosine (cytosine arabinoside). *Proc. Amer. Ass. Cancer Res.* **6,** 50 (1965). (Abstract)
8. R. W. Carey and R. R. Ellison, Continuous cytosine arabinoside infusion in patients with neoplastic disease. *Clin. Res.* **13,** 337 (1965). (Abstract)
9. K. P. Yu, J. P. Howard and B. D. Clarkson, Comparative studies of cytosine arabinoside (CA) and 5-fluoro-2′-deoxyuridine (FUdR) in leukemia in man. *Proc. Amer. Ass. Cancer Res.* **7,** 78 (1966). (Abstract)
10. M. Nesbit and J. Hartman, Cytosine arabinoside treatment of acute leukemia of childhood. *Proc. Amer. Ass. Cancer Res.* **8,** 50 (1967). (Abstract)
11. I. Kline, Denis D. Tyres, John M. Venditti, Vaman S. Waravdekar, Miriam Gang and A. Goldin, Effectiveness of cytosine arabinoside therapy on leukemia (L1210) of the central nervous system in mice arising during methotrexate treatment. *GANN* **59,** 527 (1968).
12. W. C. Werkheiser, Specific binding of 4-amino folic acid analogous by folic acid reductase. *J. biol. Chem.* **236,** 888 (1961).
13. J. J. Furth and S. S. Cohen, Inhibition of mammalian DNA polymerase by the 5′-triphosphate 1-β-D-arabinofuranosylcytosine and the 5′-triphosphate of 9-β-D arabinofuranosyladenine. *Cancer Res.* **28,** 2061 (1968).
14. Joseph H. Burchenal and Malin R. Dollinger, Cytosine arabinoside (NSC-63878) in combination with 6-mercaptopurine (NSC-755), methotrexate (NSC-740), or 5-fluorouracil (NSC-19893) in L1210 mouse leukemia. *Cancer Chemother. Rep.* **51,** 435 (1967).
15. M. Edelstein, Predicted and observed results of combination chemotherapy with inhibitors of DNA synthesis, *Proc. Amer. Ass. Cancer Res.* **17,** 59 (1973). (Abstract)
16. G. B. Grindey and C. A. Nichol, Interaction of drugs inhibiting different steps in the synthesis of DNA. *Cancer Res.* **32,** 527 (1972).
17. M. H. N. Tattersall and K. R. Harrap, Combination chemotherapy: the antagonism of methotrexate and cytosine arabinoside. *Europ. J. Cancer* **9,** 229 (1973).

18. K. D. BAGSHAWE and R. J. WOODS, Antagonism between inhibitors of DNA synthesis. *Brit. J. Cancer* **26,** 513 (1972).
19. R. R. SAKAL and F. J. ROHLFE, *Introduction to Biostatistics,* W. H. Freeman, San Francisco (1973).
20. W. J. DIXON and F. J. MASSEY, JR., *Introduction to Biostatistics* 2nd Edn., McGraw-Hill, New York (1957).
21. A. L. DELAUNOIS, *Biostatistics in Pharmacology* 1st Edn., Pergamon Press, New York (1973).

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Antitumour Evaluation of a Ribonuclease Resistant Double-Stranded RNA-Polyquaternary Ammonium Complex (BRL 10739)

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Abstract—*A double-stranded RNA (BRL 5907) isolated from a mycophage and one of its soluble polyquaternary ammonium complexes (BRL 10739) have been shown to have good activity against the well established solid L5178Y lymphoma. Good antitumour activity is also seen against the Lewis lung carcinoma on both the primary and the ensuing pulmonary metastases. In these two systems the complex BRL 10739 consistently showed superior activity to the parent nucleic acid. Excellent resistance to breakdown by pancreatic ribonuclease and by human serum was shown by the complex in direct contrast to the natural ds-RNA. The complex was shown to be more toxic in the mouse when compared with the parent material.*

INTRODUCTION

THE ANTITUMOUR activity of several natural and synthetic double-stranded RNA's in rodents is well documented [1–3]. This activity has been of sufficient interest to justify the investigation of the materials as anticancer agents in man; however, reports of human studies with the synthetic ds-RNA poly (I) .poly (C) carried out in the United States have been disappointing [4]. One reason suggested for this lack of activity is that the material can be broken down rapidly by nucleases present in human serum [5], and therefore, it is not present long enough to exert an antitumour effect.

The isolation within our laboratories of a double-stranded RNA (BRL 5907) from a virus infecting a penicillium species [6] has enabled us to evaluate the material as a potential anticancer agent. Initial activity was demonstrated against the Friend leukaemia virus [7] and a recent report has compared similarities of antitumour action of this ds-RNA with endotoxin and lipid A [8]. We wish to report the antitumour evaluation of an ionic complex of the mycophage ds-RNA which has considerable resistance to nucleases in human serum.

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MATERIAL AND METHODS

The ds-RNA complex

A number of complexes formed between ds-RNAs and quaternary ammonium polymers or polyamines has been studied within our laboratories. The soluble complex chosen for particular attention BRL 10739 was prepared by slow addition of a solution of the quaternary ammonium polymer in 0·15M sodium chloride to a 10 mg/ml solution of ds-RNA in 0·15M sodium chloride until 80% of the nucleic acid phosphate groupings were bound. The polyquaternary compound (Fig. 1) used was synthesized from tetramethylpropane-1,3-dia-

mine and 1,4-dibromobutane and contained 52 quaternary sites per polymer chain.

Nuclease studies

A solution of Seravac grade VI pancreatic ribonuclease was added to a solution of the nucleic acid in isotonic saline and the optical density of the solution at 260 nm measured at regular time intervals.

In a second study the nucleic acids were incubated for 30 min at 37°C with dilutions of human serum and then subjected to electrophoresis. The gels were scanned using an ultra violet spectrometer equipped with a linear transport scanner and the peaks integrated.

Tumour systems

The L5178Y lymphoma was maintained in the ascitic form in syngeneic DBA/2 mice [8] by serial weekly passage of washed, counted tumour cells. This lymphoma was experimentally used as a solid tumour which grew to a size of some 6 mm one week after intradermal inoculation of 10^6 cells into the flank of DBA/2 mice.

The Lewis lung carcinoma was maintained by subcutaneous inoculation into the flank of C57Bl/6 mice. The passage was carried out every 14 days as trocar fragments. The tumour was set up for experimental studies in a similar way. The tumours grew to some 3·0 g over 21 days; at this time the mice were killed and the subcutaneous tumour excised and weighed. The lungs were examined for metastases by injecting dilute Indian ink into the trachea. The lung was then removed and placed in fixation liquid where the metastases appeared as white nodules against the black substance of the lung [9, 10].

Toxicity studies

The acute study was carried out using groups of 10 CD1 mice, the nucleic acids being dosed by the intraperitoneal route. Repeat dose studies were carried out daily for 6 days again by the intraperitoneal route in CD1 mice.

RESULTS

Nuclease resistance studies

A solution of the pancreatic ribonuclease was added to a solution of BRL 5907 and BRL 10739 in isotonic saline and the optical density measured at regular time intervals. Hydrolysis of the uncomplexed ds-RNA was evidenced by a rapid onset of hyperchromicity and was complete after 5 hr (Table 1). After 18 hr no hyperchromicity was observed with the complex BRL 10739.

The ds-RNA and its complex were incubated with various dilutions of human serum and the ensuing degradation evaluated by electrophoresis. None of the intact uncomplexed

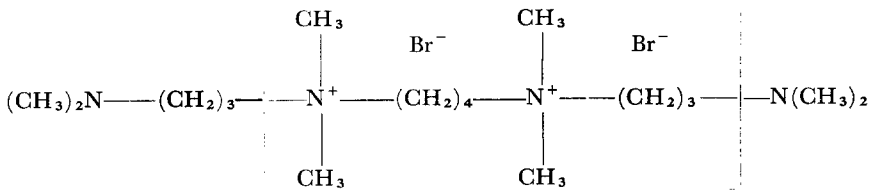


Fig. 1. *Structure of the quaternary ammonium polymer.*

Table 1. *Degradation of BRL* 5907 *and BRL* 10739 *by pancreatic ribonuclease*

Time of addn. of ribonuclease (hr)	Hyperchromicity % (260 nm) BRL 5907 (ds-RNA)	BRL 10739 (Modified ds-RNA)
0·25	8·6	0
1	25·8	0
5	46·2	0
18	46·2	0

Solution of Seravac grade VI pancreatic ribonuclease added to solution of nucleic acid (in 0·15 M saline). Optical density at 260 nm measured at regular time intervals.

material remained after exposure to a 50-fold dilution whereas the complex BRL 10739 was completely resistant to 50-, 10- and 5-fold dilutions of the serum (Table 2).

Antitumour evaluation

The complex BRL 10739 was first investigated for antitumour activity and compared with its parent nucleic acid against the L5178Y lymphoma. Good activity against this lymphoma was shown with both the complex and parent nucleic acid at 5 mg/kg when dosed on days 8, 11 and 13 and scored on day 15 (Table 3). A positive regression was scored

Table 2. Degradation of BRL 5907 and BRL 10739 by human serum

Serum dilution	% Degradation of nucleic acid	
	BRL 5907 (ds-RNA)	BRL 10739 (complex)
1/100	35	0
1/50	100	0
1/10	100	0
1/5	100	0

Nucleic acid incubated for 30 min at 37°C in dilutions of human serum. Degradation assessed by gel electrophoresis.

when complete shrinkage of the tumour had occurred. The shrinkage seen after nucleic acid treatment was normally associated with haemorrhage and scabbing of the tumour site. The tumour normally re-established itself several days after dosing was completed although "cures" were occasionally seen. On lowering the dose to 0·5 mg/kg only the complex showed activity. The activity shown by both materials was seen against the established tumour and dosing before day 8 did not produce regressions. This has been reported previously with the parent nucleic acid [7, 8]. The lack of activity against the early tumour was also noted when the materials were injected into the tumour mass; although activity was observed with high doses of the complex. The intralesion studies (Table 3) similarly demonstrated the activity of these materials against the established tumour. BRL 10739 had a better effect than ds-RNA at low dose levels.

The Lewis lung carcinoma was chosen so that the effect of these nucleic acids on both the primary tumour and the ensuing pulmonary metastases could be studied. Both the parent nucleic acid BRL 5907 and its complex BRL 10739 showed activity when dosed by the intraperitoneal route against both the primary tumour and the lung metastases (Table 4). A 66% inhibition of tumour weight compared with saline injected controls was seen with BRL 5907 when dosed at 2·5 mg/kg for 5 days each week for three weeks. BRL 10739 at 2·5 mg/kg for 2 days in each week for three weeks showed a 90% inhibition in tumour weight. The better activity shown by the

Table 4. Effect of BRL 5907 and BRL 10739 by the intraperitoneal route against the Lewis lung carcinoma

Drug and dose	Tumour Inhibition (%)	Average number of lung metastases
BRL 5907 (ds-RNA)		0·5 large
2·5 mg/kg 5 × per week	66	4·0 small
BRL 10739 (modified ds-RNA)		
2·5 mg/kg 2 × per week	90	1·0 small
Saline control		6 large
5 × per week	—	17 small

Tumour implanted day 0, subcutaneously into the flank.
Drugs given as 0·1 ml in phosphate buffered saline.
10 mice in each group.
All results significant when compared with controls ($P < 0·001$).

Table 3. Effect of BRL 5907 and BRL 10739 against the L5178Y lymphoma

Drug	Route of administration	Effect on tumour*; dose given		
		5mg/kg	2·5mg/kg	0·5mg/kg
BRL 5907	Intraperitoneal	6/6	4/6	1/6
(ds-RNA)	Intralesion	6/6	3/6	3/6
BRL 10739	Intraperitoneal	6/6	5/6	4/6
(modified ds-RNA)	Intralesion	6/6	6/6	6/6

* $\frac{\text{Number of mice showing complete tumour shrinkage}}{\text{Total number of mice in group}}$

Treatment given on days 8, 11 and 13
Effect on tumour assessed on day 15

10^6 cells were injected on day 0 intradermally into the flank.
Drugs given as 0·1 ml in phosphate buffered saline.
Intralesion or intraperitoneal injection with saline had no effect.

complex was also indicated in the lung. Thus BRL 5907 dosed mice showed a good reduction in lung metastases when compared with controls. The complex showed a significantly better result ($P<0.05$) than the parent nucleic acid with virtually no metastases in the lung.

Toxicity studies

The acute intraperitoneal toxicity of BRL 5907 and BRL 10739 was investigated in the mouse. The LD_{50} for the parent nucleic acid determined in a number of experiments was about 30 mg/kg and for the complex 20 mg/kg. Repeat dose studies in the mouse showed that BRL 5907 given by the intraperitoneal route for 6 days at 4·0 mg/kg caused profound but transient loss of bodyweight, acute lymphopenia, thrombocytopenia and the typical syndrome of ruffled coats, reduced activity and diarrhoea. On day 8 there was a neutrophil leucocytosis, reduced platelet count, reduced PCV, increased relative spleen weight and decreased relative thymus and adrenal weights. BRL 10739 given under the same regimen was clearly more toxic, 5/6 mortalities occurred during the dosing period. Measurement of the parameters mentioned above indicated a similar spectrum of toxic effects.

DISCUSSION

The antitumour properties of the natural double-stranded RNA BRL 5907 and one of its polyquaternary ammonium complexes BRL 10739 have been compared in two tumour systems. In these studies BRL 10739 has shown better activity and at lower doses than the parent material. It is likely that BRL 10739 would show good activity in the wide range of tumour systems which have been reported to be responsive to poly (I) .poly (C), the synthetic ds-RNA [1–3]. The complex BRL 10739 has also been shown to be most resistant to breakdown by human serum. Poly (I) .poly (C) is broken down quickly by human nucleases and this is a reason suggested for its poor biological activity in man [5]. It is probable that BRL 10739 will remain intact in such situations for longer periods and thus be able to exert a useful antitumour effect.

A number of antiviral protection studies with the ds-RNA and its complex have been carried out within our laboratories [11] and have indicated the superior properties of the complex BRL 10739. Preliminary studies in veterinary species to be reported in detail elsewhere [12], have also been encouraging; these suggest that the advantages of the complexed material BRL 10739 over its parent nucleic acid are greater in those species which have high levels of RNase present in their serum.

The antitumour data of the complex BRL 10739 together with its resistance to serum nucleases encourages us to think that this material may have distinct advantages over uncomplexed nucleic acids in the treatment of human neoplasms.

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REFERENCES

1. R. H. Adamson, S. Fabro, E. R. Homan, R. W. O'Gara and R. P. Zendzian, Pharmacology of polyriboinosinic: polyribocytidylic acid, a new antiviral and antitumour Agent. *Antimicrob. Agents and Chemother.* 148, (1969).
2. J. S. Rhim and R. J. Huebner, Inhibitory effect of statolon on virus-induced sarcoma of mice. *Antimicrob. Agents and Chemother.* 177, (1969).
3. H. Levy, L. W. Law and A. Rabson, Inhibition of tumour growth by polyinosinic-polycytidylic acid. *Proc. nat. Acad. Sci.* **62,** 357 (1969).
4. C. W. Young, Interferon induction in cancer. *Med. Clin. N. Amer.* **55,** 721 (1971).
5. J. J. Nordlund, S. M. Wolff and H. B. Levy, Inhibition of biologic activity of poly I poly C by human plasma. *Proc. Soc. exp. Biol* (N.Y.) **133,** 439 (1970).
6. K. W. Buck, E. B. Chain and F. Himmelweit, Comparisons of Interferon induction in mice by purified penicillium chrysogenum virus and derived double-stranded RNA. *J. Gen. Virol.* **12,** 131 (1971).
7. D. J. F. Pilch and D. N. Planterose, Effects on Friend disease of double-stranded RNA of fungal origin. *J. gen. Virol.* **10,** 155 (1971).
8. I. Parr, E. Wheeler and P. Alexander, Similarities of the antitumour actions of endotoxin, lipid A and double-stranded RNA. *Brit. J. Cancer* **27,** 370 (1973).

9. K. HELLMANN and K. BURRAGE, Control of malignant metastases by ICRF 159. *Nature (Lond.)*, **224,** 273 (1969).
10. H. WEXLER, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).
11. M. R. HARNDEN, A. G. BROWN, T. J. SHARPE and R. A. VERE-HODGE, Double stranded RNA–Quaternary ammonium polymer complexes. Potentiation of antiviral activity. 166th Meeting of the American Chemical Society, Chicago, Aug. 27th–31st, 1973.
12. R. F. SELLERS, J. W. G. LEIPER and D. N. PLANTEROSE, The effect of double-stranded RNAs on the development of foot-and-mouth disease in pigs and calves. (Personal Communication).

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Metabolism of Exogeneous Cortisol in Tumor-Bearing Rats

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Abstract—*Exogeneous cortisol disappearance rate was studied* in vivo *and* in vitro *(isolated liver perfusion and* 9000 × **g** *liver homogenate) in control and tumor bearing rats. In the tumor group an increased disappearance rate of cortisol was observed both* in vivo *and* in vitro, *together with a reduced plasma protein binding. Increased hepatic metabolism and reduced plasma protein binding do not fully explain the observed differences and other factors such as modified tissues distribution, altered renal function and modified hemodinamic situation may be involved.*

INTRODUCTION

SEVERAL reports have shown that the activity of liver drug metabolizing enzymes in experimental animals is affected by the presence of a tumor [1–3], so that the pharmacological or toxic effect of drugs is often changed in tumor bearing animals [1,4,5]. The microsomal hydroxylases of steroid hormones such as progesterone and testosterone have also been described to be altered in rats bearing Walker carcinosarcoma 256 [6].

Since cortisol and other corticosteroid are widely used in cancer therapy, it was considered of interest to investigate the influence of tumor presence on the disappearance rate of the compound and on its hepatic metabolism. This report describes studies, which have been performed *in vivo* and *in vitro*.

MATERIALS AND METHODS

Male Sprague–Dawley rats, 140 ± 5 g initial body weight, fed on a standard diet and housed in standardized conditions, were used. Walker 256 carcinosarcoma was transplanted subcutaneously in fragments of about 100 mg of a 15 days old tumor by means of a trocar into the right flank of the rats.

Experiments in vivo

Cortisol was administered at 9.30 a.m. either i.p. as free alcohol (50 mg/kg) 6 and 12 days after tumor transplantation, or i.v. as succinate half-ester† (20 mg/kg) 12 days after tumor transplantation. The control groups were animals of the same age kept in similar experimental conditions. Rats were sacrificed at different times, as indicated in Tables and plasma and tumor samples collected for cortisol determinations.

Experiments with isolated perfused liver

Rats bearing Walker 256 carcinosarcoma and/or normal animals were used as donors of livers and blood for the perfusion experiments.

The tumor bearing rats were used 12 days after subcutaneous transplantation of Walker carcinosarcoma 256. The tumor development and the general conditions of the animals were controlled accurately before the experiment. The animals used for the preparation of blood were sacrificed at 9 a.m. The liver was isolated

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†Flebocortid (Richter).

under total anesthesia (chloralose 60 mg/kg body wt., sodium phenobarbital 50 mg/kg body wt.) by the usual surgical technique and the portal vein and the biliary duct were cannulated [7].

The perfusion medium contained 1/3 defibrinated heparinized blood, 1/3 homologous serum and 1/3 Krebs–Ringer bicarbonate buffer pH 7·4. Free cortisol was dissolved in minimum quantity of ethanol (0·1 ml) and added to the perfusion medium in order to achieve the final concentration of 20 μg/ml; the total volume of medium was proportional to the liver weight i.e. 5 ml/g liver tissue. The organ was perfused by recirculation; the flow of the perfusate was 1 ml/min/per g of liver as previously described [8].

The samples of perfusion fluid were taken after 5,10,15,20 and 30 min of liver perfusion for cortisol determination.

Experiments with liver microsomal enzymes

Six and twelve days after Walker 256 carcinosarcoma subcutaneous transplantation, the rats were sacrificed at 10 a.m. and plasma collected for corticosterone determination.

For the analyses animals transplanted 12 days before with the tumor were divided in two groups: A = bearing a small tumor and B = bearing a large tumor. The livers were immediately removed and homogenized in a Potter-type Teflon homogenizer with 4 volumes of ice cold KCl 1·15% solution. The homogenates were centrifuged at 9000 × g for 20 min. Three ml of the supernatant corresponding to 600 mg of liver were then incubated with 2 ml of a phosphate buffer (pH 7·35) containing 2·3 μmoles of NADP, 58·4 μmoles of glucose-6-phosphate, 50 μmoles of nicotinamide, 25 μmoles of $MgCl_2$ and 2 μmoles of cortisol-free alcohol as substrate.

The mixture was incubated at 37°C for 25 min under air in Dubnoff metabolic shaker. The measurement of cortisol disappearance and 6-OH-cortisol formation were used to estimate the hepatic metabolism of this drug.

Drug metabolic enzyme activity was calculated as μmoles of cortisol metabolized and n-moles of 6-OH-cortisol formed per g of liver per min.

Hepatic supernatant proteins were measured by the method described by Lowry *et al.* [9] and no difference was observed between control and tumor bearing animals.

Biochemical determinations

Basal plasma corticosterone levels were determined according to Guillemin *et al.* [10].

For plasma, perfusion medium and tumor cortisol determinations the procedure was modified as follows: tumors were homogenized in ice-cold distilled water 1:5 (w/v). One ml of the homogenate was then transferred to a 50 ml centrifuge tube, diluted with 3 ml of distilled water and extracted in 12 ml of $CHCl_3$, by shaking for 30 min at room temperature. After centrifugation, 9 ml of the organic phase were transferred to another 50 ml centrifuge tube and dried under nitrogen stream or under vacuum. The same extraction procedure was also applied to plasma, to the perfusion medium and to the liver 9000 × g supernatant after incubation.

The dry residue was then redissolved in 1 ml of distilled water. The extract was partitioned between water and CCl_4 (10 ml) phases. This step allowed one to eliminate most of the interfering substances. The water phase was then diluted to 4 ml and re-extracted in 5 ml of $CHCl_3$. The purity of this extract was verified by thin-layer chromatography ($CHCl_3$–MeOH–H_2O; 23:1,5:0,15 V/V/V) system; development by 50% H_2SO_4, 10 min at 100°). The organic phase was than processed as indicated by Stockham [11]. The determinations were performed with an Aminco–Bowman spectrofluorometer (A = 470 nm; E = 525 nm) 30 min after shaking with the reagent. The over-all recovery was 83 ± 3%. The described procedure extracts less than 5% of the cortisol succinate half-ester eventually present. 6-OH-cortisol determination was performed according to Trasher *et al.* [12] with minor modifications. Statistical evaluation of the results was carried out by Student-t-test and analysis of variance.

Cortisol binding to plasma proteins

Tumor bearing animals were sacrificed twelve days after tumor transplantation. Pooled plasma samples from two animals were used. Five ml of plasma were used for each determination. After 1 hr incubation with cortisol-free alcohol (20 μg/ml), the binding was evaluated by ultrafiltration technique according to Di Salle *et al.* [13]. Plasma proteins were measured according to Lowry *et al.* [9].

RESULTS

(*a*) *Experiments* in vivo

In a first series of observations a large dose of cortisol-free alcohol (50 mg/kg) was administered intraperitoneally to tumor bearing rats and to control rats of the same age.

Table 1. Plasma levels (μg/ml) of cortisol in normal and tumor-bearing rats after an i.p. administration of cortisol-free-alcohol (50 mg/kg)

Days after transplantation	Experimental group	Body weight (g± S.E.)	Tumor weight (g± S.E.)	Time after cortisol administration 15	60	120
6	Controls	175± 5	—	14·28 (9·30–22·09)	7·64 (5·24–11·14)	1·56 (1·06–2·31)
	Walker	175± 5	0·27±0·14	6·73* (4·18–10·86)	3·25† (2·32–4·55)	0·46† (0·25–0·85)
12	Controls	212±10	—	14·83 (13·00–16·91	7·07 (5·80–8·62)	1·52 (1·03–2·26)ù
	Walker	192±10	4·9±0·4	7·82† (4·97–12·30)	3·73† (2·84–4·88)	0·46† (10·24–0·89)

* $P<0·05$.
† $P<0·01$.
Each point represent the mean of six animal—in brackets, the 95% confidence intervals.
Body weight in tumor bearing animals was calculated including the tumor weight.

Plasma cortisol levels were determined after 15, 60 and 120 min.

The means and the confidence intervals are reported in Table 1.

Marked and significant difference between the two experimental groups (controls and tumor) were present both 6 and 12 days after tumor transplantation, with cortisol levels in the tumor group of about half of those observed in controls.

In order to avoid possible interferences on drug absorption, due to a probably modified hemodynamic situation, in a second series of observations cortisol was administered intravenously, as succinate half-ester, at the dose of 20 mg/kg.

No differences in blood esterases activity were found between control and tumor bearing rats.

Plasma concentrations were determined 5, 15 and 30 min after dosing. In order to evaluate the extent of uptake by the tumor, cortisol tumor content was also measured.

From Table 2 it may be seen that although the apparent cortisol plasma half-life did not differ in the two experimental groups, in the tumor bearing animals there was a 50% increase in the apparent volume of distribution, ($Vd=D/C°$) with a total body clearance ($TBC=(Kel·Vd)$ of 0·131 ml/kg per min against a total body clearance of 0·067 ml/kg per min of the control rats. The concentration of cortisol in tumor tended to be lower than the corresponding plasma ones, suggesting a

Table 2. Plasma (μg/ml) and tissue (μg/g) cortisol concentrations in normal and tumor-bearing rats (12 days after tumor transplantation) after i.v. administration of cortisol succinate half-ester (20 mg/kg)

Experimental group	Tissue	Time after cortisol administration (min) 5	15	30	$T\frac{1}{2}$ (min)	$Vd\ \frac{l}{(kg)}$	T.B.C. (ml/kg per min)
Control	Plasma	15·25 (14·03–16·58)	7·13 (5·15–9·86)	3·04 (2·30–4·00)	10·8	1·05	0·067
Walker	Plasma)	8·10 (6·60– 9·94)	3·19 (2·13–3·79)	1·62 (0·83–3·18)	11·1	2·12	0·131
	Tumor	5·74 (4·45– 7·40)	2·97 (1·32–3·67)	0·97 (0·71–1·33)	10·0		

Each value is the mean of 5 rats. In parentheses the 95% confidence intervals.
Body weight=Controls 230±5 g. Walker 157±5 g. In tumor bearing animals the body weight was measured including the tumor weight (7·01±1·13 g).

relatively small accumulation in the neoplastic tissue. This is further supported by the fact that the apparent cortisol disappearance rate in the tumor was of the same order of the plasma one.

Evaluation of *plasma protein binding* showed that a significant decrease in binding was present in tumor bearing rats (Table 3).

The 20% increase of the free fraction is in agreement with the lower plasma protein content observed in these animals.

Table 3. Cortisol binding to plasma protein in control and tumor-bearing rats

	% Unbound	Plasma protein (g/l)
Controls	23·8 ± 0·5	62·1 ± 2·3
Walker	28·9 ± 0·6*	50·3 ± 1·7*

*$P < 0{\cdot}01$.
Each figure is the mean of seven determinations.

(*b*) *Experiments with perfused liver*

The disappearance rate of cortisol from the perfusate was studied over a period of 30 min with livers of normal and tumor bearing animals 12 days after Walker carcinosarcoma transplantation.

Cortisol concentrations in the medium decreased by a first order reaction rate in both the experimental groups.

The apparent rate constants (K_1) and the respective half-lives are reported in Table 4. With livers from tumor bearing animals the apparent $T^{\frac{1}{2}}$ was significantly reduced as compared to controls ($P < 0{\cdot}01$).

Cross experiments, i.e. perfusion of tumor liver with normal blood and vice versa, excluded that blood or serum of tumor bearing animals could somehow play a role in the modified performance of the liver of tumor bearing rats in this experimental situation.

Experiments in vivo

The disappearance rate of cortisol and formation of the 6-OH-cortisol were studied with the 9000 × *g* liver homogenate supernatant fraction of control and tumor bearing animals 6 and 12 days after Walker carcinosarcoma transplantation. The plasma corticosterone levels were 8·56 ± 1·47 μg% for the control group and 22·8 ± 1·39 μg% for the Walker group ($P < 0{\cdot}0001$). It has to be remembered that the 12 days group was divided in two sub-groups according to the tumor growth.

For each pair of control and tumor bearing animals Student's *t*-test was used to assess the significance of the difference between the mean levels of cortisol or 6-OH-cortisol.

Control values = [*Cortisol* = 62·7 ± 9·7 n-*moles/g per min*; *6-OH-Cortisol* = 6·1 ± 0·4 n-*moles/g per min*]

* P < 0·05
** P < 0·01
*** P < 0·001
in respect to conrols.

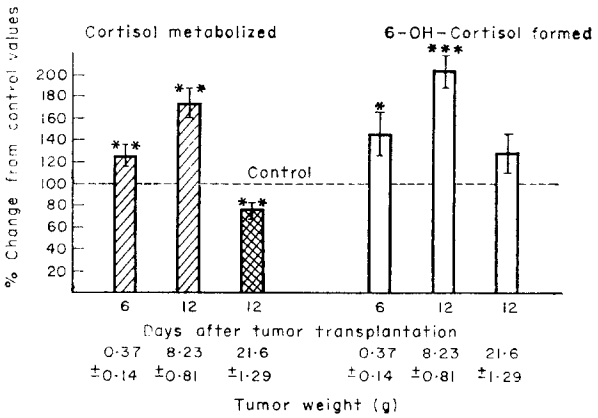


Fig. 1. Cortisol metabolism by liver homogenates (9000 × **g**) *of tumor bearing animals. The values are expressed as % of controls.*

Table 4. Cortisol disappearance rate from the perfusion medium in perfused liver preparation

Liver	Blood	$K_1 + 10^{-3}\text{min}^{-1}$ mean ± S.E.	$T^{\frac{1}{2}}$(min) mean ± S.E.
Control	Control	30·9 ± 1·7	22·4 ± 0·4
Tumor	Tumor	53·9 ± 5·7†	12·8 ± 1·2†
Control	Tumor	29·1 ± 0·5	23·8 ± 0·1
Tumor	Control	51·2 ± 3·8†	13·5 ± 1·8†

† $P < 0{\cdot}01$ in respect to control liver perfusion.
Each point is the mean of 5–7 determinations.
Liver of tumor bearing animals was taken 12 days after tumor transplantation. Liver weight = Controls 10·3 ± 0·4 g; —Tumor 12·0 ± 0·4 g. Tumor weight = 32·9 ± 1·6 g.

Since the variances for some of the pairs were not homogeneous, in these cases a modified version of the *t*-test was used according to Snedecor and Cochran [14].

The levels of significance from all these tests are reported in Fig. 1.

In control animals the disappearance rate of cortisol was $62{\cdot}7 \pm 9{\cdot}7$ *n*-moles/g per min and the formation of 6-OH-cortisol was of 6·1 *n*-moles/g per min.

The newly formed 6-OH-cortisol represented about 10% of the metabolized fraction (Table 5). In the tumor groups a significant increase of both cortisol disappearance rate and 6-OH-cortisol formations was observed with liver homogenate supernatant of the animals sacrificed 6 and 12 days after transplantation and bearing a tumor of relatively small dimension (8–10% of the body weight) (Fig. 1). In these groups the ratio between the 6-OH-cortisol formed and the cortisol metabolized did not differ significantly from the control group (Table 5).

In animals bearing a larger tumor a clear dissociation was observed between the metabolic pathways; while in fact the 6-OH-cortisol formation was still higher than in control animals, the disappearance rate of cortisol was significantly reduced, with a 64% increase of the ratio between 6-OH-cortisol formed and metabolized ($P < 0{\cdot}01$).

In addition, $9000 \times g$ preparation from liver of rats and mice bearing different experimental tumors metabolize zoxazolamine and other substrates to a reduced rate when compared to controls [1,16,17].

Similar findings have been reported by Kato [2, 4] and Wilson [13, 18] in *in vivo* and *in vitro* studies. For all the substrates investigated there seems to be positive relationship between impairment of hepatic drug metabolism and tumor growth.

With regard to the steroid hormones, which are physiological substrates for the microsomal enzymes, Kato [6] described a reduced hydroxylation and an increased production of non polar metabolites by incubating testosterone and progesterone with microsomal preparation from liver of tumor bearing animals.

Our results with cortisol are apparently in partial disagreement with the data obtained with other substrates. In fact only when the tumor weight approached 20 g and the animals were in a cachexia condition we observed a reduced disappearance rate of cortisol *in vitro*.

During earlier stages of the tumor growth both the data *in vitro* (utilizing the $9000 \times g$ preparation or the perfused liver technique) and *in vivo* show an increased disappearance rate of cortisol in tumor bearing rats when compared to controls.

An increased catabolism of cortisol is supported by the *in vitro* data and by the increased production of 6-OH-cortisol.

In this regard an increased urinary output of 6-OH-cortisol in presence of tumor has been previously observed in humans [19] and in guinea pigs [20]. In our experimental conditions, in agreement with previous reports [20–22], we observed that in all the groups studied there was a relevant increase of endogeneous corticosterone plasma levels. This rise

Table 5. Ratio between 6-OH-cortisol formed and cortisol metabolized by liver 9000 × g preparations of control and tumor-bearing rats

Experimental groups		$\frac{\text{60-H-cortisol formed}}{\text{cortisol metabolized}} \times 100$
Control		9·7
Walker 6 days	Tumor weight (0·37 g)	10·9
Walker 12 days	Tumor weight (8·20 g)	11·3
Walker 12 days	Tumor weight (21·62 g)	15·9*

* $P < 0{\cdot}01$ in respect to control group.

DISCUSSION

An alteration of drug metabolism in tumor bearing animals has been reported by several authors. Previous studies from this group [1,15,16] showed that pentobarbital sleeping time and zoxazolamine paralysis were markedly prolonged in tumor bearing rats, concomitantly with drug levels in blood and brain higher than in control rats.

was already present few days after tumor transplantation.

Since it was suggested [23] that enhanced levels of blood corticosteroid may determine an increased metabolization of these substrates, the increased disappearance rate of cortisol observed during liver perfusion and with the $9000 \times g$ preparation could partially be explained by this factor.

Another evidence that liver activities are implied is brought by the cross over experiments with isolated perfused liver, where at variance with what observed for other substrates [24] blood or serum factors did not influence the clearance of the drug.

An increased metabolism, however, does not seem to be the only factor involved when we consider the *in vivo* data.

First of all the volume of distribution is significantly different between tumor bearing and control rats. The additional compartment (the tumor), in equilibrium with plasma, could in part explain the lower plasma levels observed at 5 min after the i.v. administration. At that time, assuming an even distribution, the tumor mass is in fact sequestrating about 39·9 μg of cortisol equivalent to 13% of the administered dose. A different blood esterases-activity in hydrolizing the succinate half-ester can be excluded, since no differences were observed by incubating cortisol succinate half-ester with plasma and blood of tumor bearing and control rats.

Secondly the reduced protein binding observed in tumor bearing animals, in agreement with previous data of Jensen [25] and Bell [26] in cancer patients, leading to a 21% increase in the cortisol free fraction may also have some bearing on the increased drug clearance observed *in vivo*.

Furthermore a modified renal clearance of the compound has to be considered since data of Dinh [27] describe a marked renal lesion in Walker tumor bearing rats. Finally the possibility that cortisol may be degraded by tumoral tissues cannot be excluded in the light of the data reported by Haley *et al.* [28] and Kemeny *et al.* [29].

By a general point of view we think the data described here, together with the ones previously reported by various authors, underline once again that drugs kinetic may be altered in tumor bearing animals. Many factors (modification of hepatic metabolism, protein binding, distribution, renal excretion, hemodinamic situation and nutritional status) may play a role in the *in vivo* situation.

The final result, consisting in a decrease or an increase drug clearance, depend on the relative bearing of the various factors.

The fact that the tumor presence may modify the drug clearance may have important reflection at a clinical level, and stresses the need of more kinetic studies in cancer patients, since the assessment of drug plasma levels together with a pharmaco-kinetic approach is to-day the basis for a more rationale therapy.

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REFERENCES

1. R. Rosso, M. G. Donelli, G. Franchi and S. Garattini, Impairement of drug metabolism in tumor-bearing animals. *Europ. J. Cancer* **7,** 565 (1971).
2. 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).
3. 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).
4. 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).
5. B. M. Boulos and C. Sirtori, Barbiturate metabolism as affected by neoplastic disease. *Clin. Toxicol.* **4,** 361 (1971).
6. R. Kato and A. Takahashi, Decreased hydroxylation of steroid hormones by liver microsomes from rats bearing Walker carcinosarcoma 256. *Cancer Res.* **30,** 2346 (1970).
7. J. Květina and Guaitani, A versatile method for the *in vitro* perfusion of isolated organs of rats and mice with particular reference to liver. *Pharmacol. (Basel)* **2,** 65 (1969).

8. I. Bartošek, A. Guaitani and S. Garattini, Long-term perfusion of isolated rat liver. *Pharmacol. (Basel)* **8,** 244 (1972).
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. R. Guillemin, G. W. Clayton, H. S. Lipscomb and J. D. Smith, Fluorometric measurement of rat plasma and adrenal corticosterone concentration. *J. Lab. clin. Med.* **53,** 830 (1959).
11. M. A. Stockham, The assay of cortisol and corticosterone in human plasma. *J. Endocr.* **26,** iv (1963).
12. K. Thrasher, E. E. Werk, Y. Choi, L. J. Sholiton, W. Meyer and C. Olinger, The measurement, excretion, and source of urinary 6-hydroxycortisol in humans. *Steroids* **14,** 455 (1969).
13. E. Di Salle, G. M. Pacifici and P. L. Morselli, Studies on plasma protein binding of carbamazepine, *Pharm. Res. Pomm.* **6,** 193, (1974).
14. G. W. Snedecor and W. G. Cochran, *Statistical Methods*, 6th Edn. Iowa State University Press, Ames, Iowa (1967).
15. R. Rosso, E. Dolfini and M. G. Donelli, Prolonged effect of pentobarbital in tumor-bearing rats. *Europ. J. Cancer* **4,** 133 (1968).
16. G. Franchi and R. Rosso, Metabolic fate of zoxazolamine in tumor-bearing rats. *Biochem. Pharmacol.* **18,** 236 (1969).
17. R. Rosso, E. Dolfini and G. Franchi, Metabolism of amphetamine in tumor-rats. *Biochem. Pharmacol.* **17,** 633 (1968).
18. J. T. Wilson, Altered rat hepatic drug metabolism after implantation of a pituitary mammotropic tumor (MtT), Walker carcinosarcoma or adenocarcinoma and after removal of the MrT. *Endocrinology* **88,** 185 (1971).
19. E. E. Werk, J. MacGee and L. J. Sholiton, Altered cortisol metabolism in advanced cancer and other terminal illnesses: excretion of 6-hydroxycortisol. *Metabolism* **13,** 1425 (1964).
20. E. M. Nadel and S. Burstein, Urinary excretion of corticosteroids in guinea-pigs with malignant neoplastic diseases. *J. nat. Cancer Inst.* **17,** 213 (1956).
21. W. Van Hove and A. Vermeulen, Plasma cortisol levels in bronchial carcinoma. Influence of cytostatic treatment. *Rev. Europ. Etud. clin. Biol.* **15,** 96 (1970).
22. V. Jensen, N. Deshpande, R. D. Bulbrook and T. W. Doouss, 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).
23. T. Uete and A. J. Jr. Blair, Autoregulatory system of corticosteroid metabolism in the liver. *Metabolism* **14,** 904 (1965).
24. I. Bartošek, A. Guaitani and M. G. Donelli, Prolonged metabolism of pentobarbital in isolated perfused liver of tumor-bearing rats. *Biochem. Pharmacol.* **21,** 2359 (1972).
25. V. Jensen, N. Deshpande, R. D. Bulbrook and T. W. Doouss, Adrenal function in breast cancer: the distribution of cortisol in patients with early or advanced breast cancer and in normal women. *J. Endocr.* **42,** 433 (1968).
26. E. Bell, R. D. Bulbrook and N. Deshpande, Transcortin in the plasma of patients with breast cancer. *Lancet* **ii,** 395 (1967).
27. B. -L. Dinh and A. Brassard, Renal lesions associated with the Walker 256 adenocarcinoma in the rat. *Brit. J. exp. Path.* **49,** 145 (1968).
28. H. B. Haley, D. F. Dimick and M. B. Williamson, Corticosteroid metabolism by human breast tumor tissue. *Surg. Gynec. Obstet.* **123,** 812 (1966).
29. V. Kemény, K. Farkas, A. Körösi and T. Szarvas, The cortisol metabolism of human pulmonary tumour tissue. *Experientia* **24,** 611 (1968).

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Plasma Prolactin, GH, LH, FSH, TSH and Testosterone During Treatment of Prostatic Carcinoma with Oestrogens*

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Abstract—*Fourteen patients with prostatic carcinoma were treated with various doses of diethylstilboestrol (DES) and Premarin (conjugated equine oestrogens). DES (15 mg/day) produced a significant increase in plasma concentrations of growth hormone and prolactin and a decrease in plasma concentrations of testosterone, luteinizing hormone and follicle stimulating hormone. Premarin (15 mg/day) reduced plasma concentrations of testosterone and luteinizing hormone but had no effect on plasma concentrations of growth hormone and prolactin. Plasma levels of thyroid stimulating hormone were unaffected by treatment. The oral administration of glucose reduced plasma levels of prolactin.*

INTRODUCTION

THE EARLY work of Huggins and his colleagues showed that the growth of prostatic carcinoma is stimulated by androgens, and is inhibited by oestrogens [1]. Oestrogen therapy lowers plasma concentrations of testosterone and its success appears to be related to the depressive effects on androgen metabolism in general [2–4]. Although the majority of cases respond to treatment, recurrence frequently occurs and there is evidence that this is accompanied by a gradual rise in plasma levels of testosterone [5]. This may result from stimulation of the testis or adrenal.

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Oestrogen increases adrenal weight in the rat in addition to lowering plasma concentrations of luteinizing hormone and raising circulating levels of prolactin and growth hormone [6, 7]. Prolactin has been shown to stimulate the release of testosterone by the human adrenal *in vitro* [8] and it may have an ancillary function in maintaining normal androgen synthesis by the testis [9]. However, no studies have yet been reported on the effects of long-term oestrogen treatment on plasma levels of prolactin in the human male.

A previous investigation was made by some of us into the changes in carbohydrate and lipid metabolism during the oestrogen therapy of prostatic cancer [10]. Plasma samples were obtained during glucose tolerance tests as part of that study. In the work reported here, we have used the same samples to examine the

effect of treatment with diethylstilboestrol (DES) and Premarin (conjugated equine oestrogens) on plasma levels of prolactin and other pituitary hormones in patients with carcinoma of the prostate.

MATERIAL AND METHODS

Fourteen patients with histologically-proven carcinoma of the prostate were studied. Their ages ranged from 49 to 83 years (mean 71 years). The patients had the nature of the study explained to them and had agreed to take part; they were treated with either DES (7·5 or 15 mg/day) or Premarin (15 mg/day). One patient was also treated with DES (1 mg/day). Not every patient underwent all the treatment regimens and the order in which the drugs were administered was varied. Six patients were also studied before starting therapy.

With most patients, 50-g oral glucose tolerance tests were carried out during a short hospital stay; the remainder underwent the tests as outpatients. The tests were started between 6·00 and 9·00 hours, blood being taken at 0, 30, 60 90 and 120 min after glucose ingestion.

One or more glucose tolerance tests were carried out after a patient had been on a particular treatment regime for 6–30 weeks.

Plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double antibody radioimmunoassays shown to be specific for these hormones [11]. The sensitivity of each assay was 0·5 m.i.u./ml of 2nd IRP HMG and MRC 63/15 respectively. Plasma thyroid stimulating hormone (TSH) was measured by a double antibody radioimmunoassay using reagents distributed by the Medical Research Council. In this assay system, negligible cross-reaction was observed with FSH, LH, growth hormone and prolactin. The sensitivity of the assay was 0·5 μu/ml of MRC 68/38 TSH. Plasma growth hormone (GH) was estimated by a specific double antibody radioimmunoassay [12] using purified human growth hormone (MRC 69/46) and rabbit antiserum to human growth hormone (Wellcome Reagents Ltd). Plasma prolactin was measured by a homologous double antibody radioimmunoassay in which there was negligible cross-reaction with FSH, LH, TSH, GH and chorionic somatomammotrophin [13]. The sensitivity of the assay was 10 milliampoules/ml of MRC 71/222. Total plasma testosterone was measured by a specific radioimmunoassay, the sensitivity of the method being 0·015 ng/ml [14].

Statistical analyses were performed by non-parametric methods.

RESULTS AND DISCUSSION

Oestrogen treatment produced a fall in plasma testosterone concentrations (Table 1). However, measurable amounts of the hormone were still detectable in the plasma even at the highest dose of DES. Similar findings were reported by Mackler *et al.* [15], showing that

Table 1. Effect of diethylstilboestrol (DES) and Premarin on plasma concentrations of prolactin, growth hormone, testosterone, luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone in patients with prostatic carcinoma

Treatment	T	GH	PRL	LH	FSH	TSH
Nil	5·47 (3·70–13·12) $N=7$	6 (2–23) $N=6$	28 (12–37) $N=6$	3 (2–5) $N=5$	2 (0–21) $N=5$	1 (0–2) $N=5$
DES 7·5 mg/day	[e]0·60 (0·29–0·73) $N=5$	17·5 (11–25) $N=6$	23 (21–76) $N=6$	—	—	—
DES 15 mg/day	[e]0·54 (0·41–2·75) $N=5$	[c]24 (11–50) $N=9$	[c]45 (23–68) $N=9$	[b]1 (0–3) $N=8$	[a]0 (0–2) $N=8$	2 (0–6) $N=8$
Premarin 15 mg/day	[e]0·70 (0·48–0·98) $N=4$	17 (5–50) $N=8$	35·5 (16–88) $N=8$	[d]1 (1–1) $N=5$	0 (0–1) $N=5$	2 (1–5) $N=5$

T, testosterone (ng/ml); GH, growth hormone (μu 1st IRP HGH/ml); PRL, prolactin (milliampoules MRC 71/222/ml); LH, luteinizing hormone (m.i.u. MRC A 63/15/ml); FSH, follicle stimulating hormone (m.i.u. 2nd IRP-HMG/ml; TSH, thyroid stimulating hormone (μu MRC 68/38/ml); *N*, number of patients. Values shown are the median and range of hormone concentrations found in plasma taken from patients after an overnight fast. *P* (*v.* controls): a<0·05; b<0·03; c<0·02; d<0·008; e<0·002; (Mann–Whitney two-tailed *U*-test).

Table 2. Effect of glucose on plasma concentrations of prolactin (PRL), growth hormone (GH) and testosterone (T) in patients with prostatic cancer (all treatments)

Hormone	Minutes after glucose ingestion			
	30	60	90	120
PRL	95(53–164) $N=25$	c86(51–172) $N=26$	a86(57–182) $N=25$	b90(46–173) $N=25$
GH	100(13–860) $N=26$	86(9–420) $N=26$	79(9–400) $N=26$	84(11–500) $N=25$
T	109(39–169) $N=17$	82(41–152) $N=17$	86(20–205) $N=16$	b80(38–124) $N=16$

Values represent the hormone concentration (median and range) at each time interval expressed as a percentage of the hormone concentration at 0 min. P (*v.* 0 min): a<0·03; b<0·007; c<0·004; (Wilcoxon two-tailed matched-pairs signed-ranks test).

large doses of oestrogen, while significantly suppressing LH and FSH release, do not succeed in completely abolishing testosterone synthesis.

It was of interest that the highest doses of DES (15 mg/day) stimulated growth hormone secretion, whereas the lower doses of DES and Premarin were without effect (Table 1). Premarin has only about half the oestrogenic activity of DES [16], probably accounting for the failure of the former drug to influence GH secretion. Although oestrogen has been shown to enhance the GH response to an infusion of arginine [17], our results (example Fig. 1) indicate that basal plasma levels of GH also increase after long-term administration of the drugs. Growth hormone has a powerful lipolytic action [18] and the stimulation of GH secretion may be responsible for the rise in plasma triglycerides that is observed during oestrogen therapy [10, 19].

Studies in women with breast cancer have shown that prolactin concentrations in the plasma rise after treatment with DES [20]. A similar increase in circulating prolactin was demonstrated in our patients treated with DES (15 mg/day). In contrast, DES (7·5 mg/day) or Premarin (15 mg/day) had no significant effect on plasma prolactin levels (Table 1).

The higher dose of DES (15 mg/day) and Premarin (15 mg/day) both significantly lowered LH concentrations in the plasma (Table 1). This confirms the reports of earlier workers [2, 5]. We also observed a reduction in plasma levels of FSH (Table 1), as has been noted in castrated human males after the administration of DES [21]. On the other hand, plasma concentrations of TSH were unaffected by oestrogens (Table 1). Such a finding was not unexpected as animal experiments suggest that oestrogens do not have a consistent effect on TSH secretion [22].

After glucose ingestion, plasma levels of prolactin fell by comparison with the fasting values (Table 2). A significant reduction in plasma levels of testosterone was also detected 120 min after glucose administration (Table 2). Since glucose is known to inhibit the secretion of GH and testosterone [23], these results indicate the possibility of a similar effect on prolactin secretion.

Prolactin and LH may have a synergistic action on testosterone synthesis in the testis

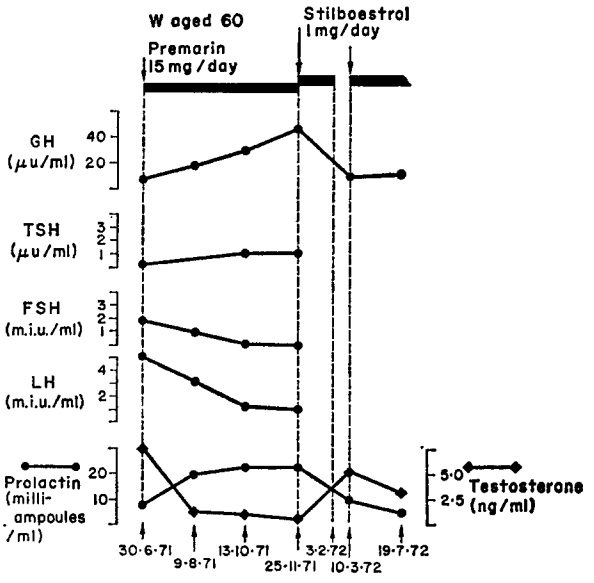


Fig. 1. Plasma hormone levels in a patient with prostatic carcinoma being treated with oestrogens. GH, growth hormone; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

[9], while the addition of prolactin to organ cultures of human adrenal tissue results in a stimulation of androgen synthesis [8]. It is therefore possible that the raised plasma concentration of prolactin contributes towards the gradual increase in plasma levels of testosterone that is often observed in patients on oestrogen therapy.

Although excessive stimulation of GH and prolactin secretion may be avoided by using doses of DES of less than 15 mg/day, consideration must now be given to the use of anti-prolactin drugs (e.g. 2-Br α-ergocryptine) as part of the endocrine therapy of prostatic carcinoma. Such studies are now being undertaken.

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REFERENCES

1. C. Huggins, W. W. Scott and C. V. Hodges, Studies on prostatic cancer*III*. The effects of fever, of deoxycorticosterone and of estrogen on clinical patients with metastatic carcinoma of the prostate. *J. Urol.* **46,** 997 (1941).
2. A. Alder, H. Burger, J. Davis, A. Dulmanis, B. Hudson, G. Sarafaty and W. Straffon, Carcinoma of prostate: response of plasma luteinizing hormone and testosterone to oestrogen therapy. *Brit. med. J.* **i,** 28 (1968).
3. W. R. Slaunwhite, A. A. Sandberg, J. E. Jackson and W. J. Staubitz, Effects of estrogen and HCG on androgen synthesis by human testis. *J. clin. Endocr.* **22,** 992 (1962).
4. M. Oshima, K. Wakabayashi and B. Tamaoki, The effects of synthetic oestrogen upon the biosynthesis *in vitro* of androgen and luteinizing hormone in the rat. *Biochim. biophys. Acta* **137,** 356 (1967).
5. M. R. G. Robinson and B. S. Thomas, Effect of hormonal therapy on plasma testosterone levels in prostatic carcinoma. *Brit. med. J.* **ii,** 391 (1971).
6. H. M. Lloyd, J. D. Meares and F. J. Thomas, Duration and nature of altered pituitary function in the male rat after a single large dose of estrogen. *Horm. Metab. Res.* **4,** 392 (1972).
7. V. Danutra, M. E. Harper, A. R. Boyns, E. N. Cole, B. G. Brownsey and K. Griffiths, The effect of certain stilboestrol analogues on plasma prolactin and testosterone in the rat. *J. Endocr.* **57,** 207 (1973).
8. A. R. Boyns, E. N. Cole, M. P. Golder, V. Danutra, M. E. Harper, B. Brownsey, T. Cowley, G. E. Jones, and K. Griffiths, Prolactin studies with the prostate. In *Prolactin and Carcinogenesis.* (Edited by A. R. Boyns and K. Griffifths) p. 207, Alpha Omega Alpha Publishing, Cardiff (1972).
9. A. A. Hafiez, C. W. Lloyd and W. Bartke, The role of prolactin in the regulation of testis function: the effects of prolactin and luteinizing hormone on the plasma levels of testosterone and androstenedione in hypophysectomized rats. *J. Endocr.* **52,** 327 (1972).
10. M. Shahmanesh, C. H. Bolton, R. C. L. Feneley and M. Hartog, Metabolic effects of oestrogen treatment in patients with carcinoma of prostate: a comparison of stilboestrol and conjugated equine oestrogens. *Brit. med. J.* **ii,** 512 (1973).
11. G. V. Groom, M. A. Groom, I. D. Cooke and A. R. Boyns, The secretion of immuno-reactive luteinizing hormone and follicle stimulating hormone by the human foetal pituitary in organ culture. *J. Endocr.* **49,** 335 (1971).
12. D. S. Schalch and M. L. Parker, A sensitive double antibody immunoassay for human growth hormone in plasma. *Nature* (*Lond.*) **203,** 1141 (1964).
13. E. N. Cole and A. R. Boyns, Radioimmunoassay for human pituitary prolactin, using antiserum against an extract of human amniotic fluid. *Horm. Res.* **4,** 261 (1973).
14. S. G. Hillier, B. Brownsey and E. H. D. Cameron, Some observations on the determination of testosterone in human plasma by radioimmunoassay using antisera raised against testosterone-3-BSA and testosterone-11α-BSA. *Steroids* **21,** 735 (1973).
15. M. A. Mackler, J. P. Liberti, M. J. V. Smith and G. R. Prout, Effect of orchiectomy and various doses of stilbestrol on plasma testosterone levels in patients with carcinoma of the prostate. *Surg. Forum.* **21,** 540 (1970).
16. S. L. Israel, *Diagnosis and Treatment of Menstrual Disorders and Sterility.* 5th Edn., Evanston, New York (1967).

17. A. G. Frantz and M. T. Rabkin, Effects of estrogen and sex difference on secretion of human growth hormone. *Endocrinology* **75,** 1470 (1965).
18. W. M. Hunter, W. M. Rigal and M. Y. Sukkar, Plasma growth hormone during fasting. In *Growth Hormone* (Edited by A. Pecile and E. E. Müller) p. 408. International Congress Series No. 158, Excerpta Medica Foundation, Amsterdam, (1968).
19. M. Kontturi and E. Sotaniemi, Effect of estrogen on the serum cholesterol and triglyceride levels of prostatic cancer patients. *J. Urol.* **105,** 847 (1971).
20. A. P. M. Forrest, Introduction to prolactin, the pituitary and breast cancer. In *Prolactin and Carcinogenesis.* (Edited by A. R. Boyns and K. Griffiths) p. 124, Alpha Omega Alpha Publishing, Cardiff (1972).
21. W. D. Odell, A. F. Parlow, C. M. Cargille and G. T. Ross, Radioimmunoassay for human follicle-stimulating hormone: physiological studies. *J. clin. Invest.* **47,** 2551 (1968).
22. J. S. Fisher and S. A. D'Angelo, Stimulatory and inhibitory action of estradiol on TSH secretion. *Endocrinology* **88,** 687 (1971).
23. J. R. Wall, R. J. Jarrett, P. Z. Zimmett, M. Bailes and C. M. Ramage, Fall in plasma–testosterone levels in normal male subjects in response to an oral glucose load. *Lancet* **i,** 967 (1973).

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Relationship Between the Growth Rate of Human Metastases, Survival and Pathological Type

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Abstract—*Data have been collected for* 203 *patients on the growth rate of pulmonary metastasis and survival, defined as delay between the diagnosis of the primary tumour and death. These cancers when found to be disseminated were not treated. The distribution of the survival times was found to be log-normal. The survival of the patient increased as the doubling time (D.T.) of the tumour increased. This group of patients was subdivided according to five histological types. A similar correlation between survival and doubling time was found in the five sub groups.*

As previously shown, the histologic subgroups have different mean D.T., but it was found that the increase of mean survival from one subgroup to another is shorter that what would be expected from the increase of D.T. Furthermore, for the same D.T., survival is longer for patients with a cancer of the histologic type with the shortest mean D.T.

Assuming the growth rate of the tumours remain constant, it is possible to calculate the number of times the volume of the tumour doubles during the survival time. When comparing the various histological subgroups, this mean number increases as the mean growth rate becomes more rapid.

INTRODUCTION

PREVIOUS works on experimental tumours [1, 2] have shown a relationship between growth rate and survival time in animals. The death of the animal is attributed to the presence of a critical number of cancer cells. The delay between the implantation of a certain number of cancer cells and the time when this critical number is reached is dependent on the growth of the tumour [3].

The growth rate of human tumours is slower than for transplanted tumours in small rodents or spontaneous tumours in dogs [4, 5]. In addition, the ratio of cancer cells to normal cells is smaller than in experimental tumours [6]. In spite of these differences, it was thought useful to investigate the relationship between the growth rate of the tumours and the survival of the patients. This is the aim of this paper.

In a previous paper [7], the existence of a relationship between growth rate and the histological type of human tumours was shown, using the doubling times (D.T.) of human metastases as measured by various authors. The study of the relationship between growth rate and survival time is also based on human metastasis doubling times. The results obtained lead to a discussion of the biological meaning of survival, that is the cause of death in cancer patients.

In addition, the relationship between the age of the patient and the growth rate of the tumour has been studied.

MATERIAL AND METHODS

Data on 530 patients were collected from the literature [7]. Patients satisfying certain con-

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ditions were selected from this group. These were patients with at least one pulmonary metastasis for which the doubling time (D.T.) has been measured; who had received no treatment likely to alter the growth rate of the tumour; for whom the histological type of the primary tumour, the age of the patient at diagnosis and the time interval between diagnosis and death was known.

Two hundred and three patients fulfilled these requirements, as summarised in Table 1.

This collection of patients could be subdivided into 5 classes based on histological type, being 12 hematosarcomas, 16 embryonal carcinomas, 63 mesenchymal sarcomas, 30 squamous cell carcinomas and 82 adenocarcinomas. The mesenchymal sarcomas were further subdivided into 22 fibrosarcomas and 41 other mesenchymal sarcomas which were studied separately.

The different correlations (notably between D.T. and survival) were calculated using the formula:

formula: $$r = \frac{\text{Covar}\ (X, Y)}{\sqrt{\text{Var.}\ X \,.\, \text{Var.}\ Y}}$$

RESULTS

1. *The distribution of individual survival times*

A. The individual survival times for the 203 cases are distributed according to a log-normal function (Fig. 1). The cumulative percentage of survival plotted against survival time is a straight line in log-probit coordinates (Fig. 2).

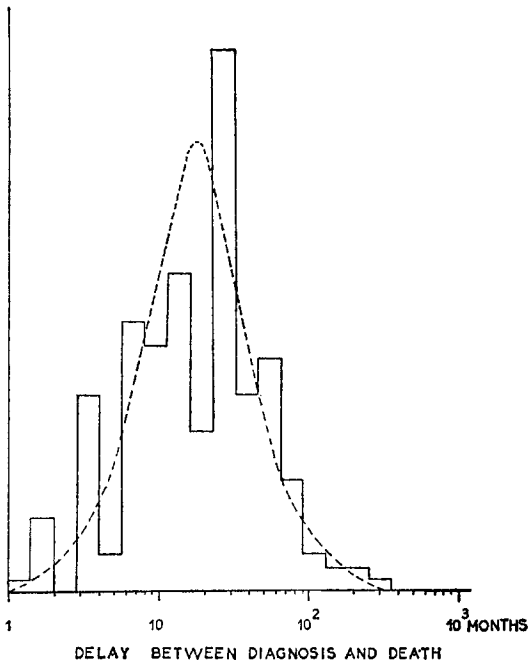


Fig. 1. Histogram of the percentage distribution of the tumours as a function of the delay between diagnosis and death.

The regression line for these points is:

$$Y = (2{\cdot}26 \pm 0{\cdot}08)X + b$$

The (geometric) mean value for survival is 17·4 months. The dispersion of individual results is very large since there is a factor of 54 between the values of the 95% confidence limits (2·5 –126 months). A spontaneous survival longer than 20 years was observed in 0·5% of cases. Extrapolation of the line suggests that a survival longer than 35 years should be

Table 1. Breakdown by author, pathological type, tissue of origin of the different groups of patients studied

Authors	Embryonal carcinomas	Hemato-sarcomas	Mesenchymal sarcomas (Fibrosarc. excluded)	Fibro-sarcomas	Squamous cell carcin.	Adenocar-cinomas	Total
Collins [8, 9]	—	—	—	—	—	22	22
Breur [10]	10	3	24	16	11	19	83
Combes *et al.* [11]	2	4	11	3	19	41	80
Rambert *et al.* [12]	4	5	6	3	—	—	18
Total	16 11 test. tum. 3 chorioc. 1 ovar. tum.	12 9 reticular cell sarc. 2 histios. 1 lymphom.	41	22 14 E.N.T. 4 cervix uter. 12 unclass.	30 38 gastro-intest. tract. 17 thyr. gland 11 breast 6 parot. gland 6 corpus uterii 3 kidney 1 ovary	82	203

observed in about 0·1% of the patients excluding cases of death other than cancer.

The distribution of the D.T. for these 203 patients is log-normal (Fig. 2). The mean value is 53·7 days. The regression line (on log-probit paper) is similar to that obtained for survival time, being:

$$Y = (2{\cdot}57 \pm 0{\cdot}06)X + b$$

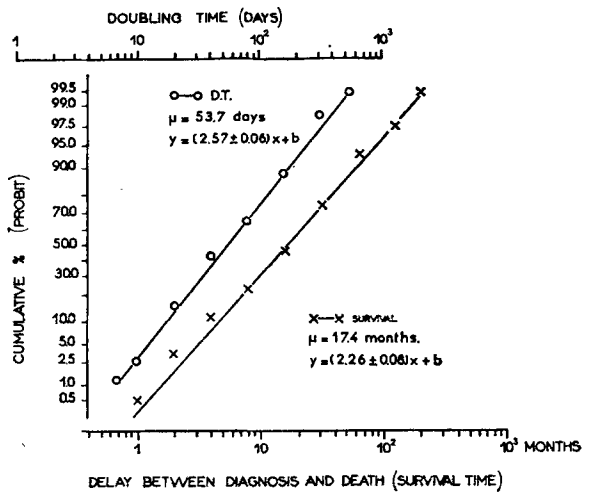


Fig. 2. Distribution of the cumulative percentages of the total number of tumours as a function of the D.T. and the delay between diagnosis and death.

B. The mean survival time of those patients with slowly growing tumours (i.e. adenocarcinomas and fibrosarcomas) is significantly longer than for those with rapidly growing tumours (embryonal carcinomas and hematosarcomas) (Table 2).

The distribution of individual values does not vary significantly for different histological types with the exception of the fibrosarcomas (Table 2—Fig. 3) within each histological subgroups, the distribution of individual values follows the equation:

$$Y = 2{\cdot}33\ X + b$$

2. *The correlation between the D.T. of the metastases and the survival time*

A. The correlation between these two parameters has been studied for each of the histological subgroups and for the whole group (Table 2). All the correlations except for embryonal carcinomas were found to be very

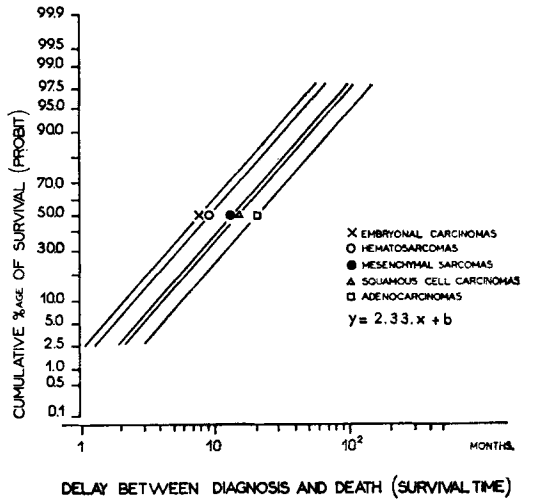


Fig. 3. Distribution of the cumulative percentages of the tumours within the different pathological groups as a function of the delay between diagnosis and death.

Table 2. Survival time of the patients, D.T. of the lung metastasis, distribution of the values of the survival time (log. surv. time = a. log. D.T. + b) (see Fig. 3) and correlation coefficient between D.T. and survival time

Pathological groups	Survival time of the patients (geometric mean and confidence interval)	Doubling time of the lung metastas. (geometric mean)	*a* (slope) (mean and confidence interval)	Correlation coefficient between D.T. and survival time	Level of significance
Embryonal tumors	9·5 months (6·7–13·6)	19·5 days	2·94 (2·23–3·65)	0·45	0·10 (NS)
Hematosarcomas	10·5 (6·2–17·8)	29·5	2·34 (1·62–3·06)	0·87	0·01
Mesenchymal sarcomas	15·5 (11·8–20·4)	35·6	2·68 (2·40–2·96)	0·51	0·01
Squamous cell carcinomas	15·6 (10·9–22·2)	51·0	2·26 (1·91–2·62)	0·45	0·01
Adenocarcinomas	20·4 (16·3–25·7)	90·4	2·22 (2·07–2·36)	0·64	0·01
Fibrosarcomas	35·7 (22·2–57·6)	64·9	2·03 (1·61–2·44)	0·49	0·01

significant. The survival time of the patient increases as the D.T. of the tumour increases.

B. With the mean value of the D.T. plotted on the abcissa and the mean value of the survival plotted on the ordinate, each histological group may be represented by a point. These points define a straight line whose slope indicates that, when various histological types are compared, the relationship between D.T. and survival time is more complex than proportionality (Fig. 4).

3. *The correlation between the D.T. and the multiplication coefficient of the tumour during the survival of the patient*

Since the tumour D.T. and the survival time are known for each patient, it is possible to calculate what we have called the multiplication coefficient of tumour cells, being the number of times the tumour doubles during the survival time assuming that the growth rate remains constant. The multiplication coefficient is equal to the survival time divided by the D.T.

Apart from the fibrosarcomas, the mean multiplication coefficient increases for the various histological groups, as the D.T. becomes shorter (Table 3—Fig. 5).

The correlation coefficients between the tumour D.T. and the number of doublings during the survival of the patients has been

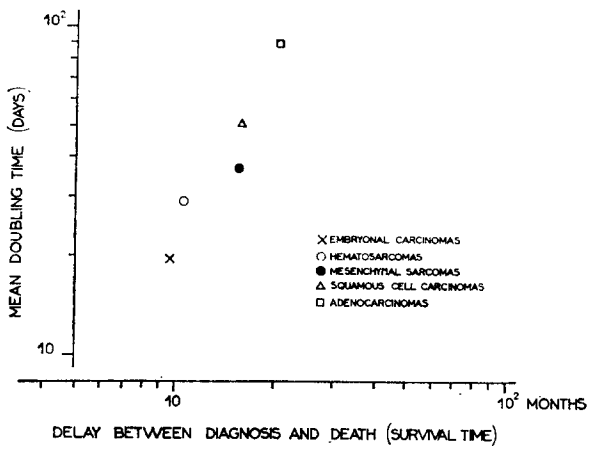


Fig. 4. Relationship between the mean D.T. and the mean delay between diagnosis and death within the different pathological groups.

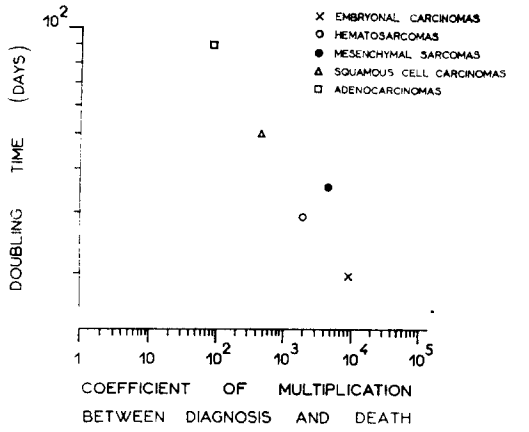


Fig. 5. Relationship between the mean D.T. and the mean coefficient of multiplication between diagnosis and death within the different pathological groups.

The coefficient of multiplication is obtained with the formula: delay between diagnosis and death/D.T. of the tumour.

Table 3. Mean values of the coefficient of multiplication of the population of tumour cells during the survival time of the patients; coefficient of correlation between D.T. and coefficient of multiplication

Pathological groups	Coefficient of multiplication	D.T. of the lung metastasis (days)	Correlation coefficient between log. D.T. and log. coefficient of multiplication	Level of significance
Embryonal tum.	$2.^{13\cdot3}$	19·5	−0·70	0·01
Hematosarcom.	$2.^{11\cdot0}$	29·5	−0·19	N.S.
Mesenchymal sarcomas	$2.^{12\cdot4}$	35·6	−0·28	0·10
Squamous cell carcinomas	$2.^{9\cdot0}$	51·0	−0·22	N.S.
Adenocarcin.	$2.^{6\cdot6}$	90·4	−0·05	N.S.
Fibrosarcomas	$2.^{16\cdot5}$	64·9	−0·27	N.S.
203 tumours	—	—	−0·31	0·01
181 tumours (203 tumours, fibrosarcom. excluded)	—	—	−0·35	0·01

calculated for each histological type and for the whole group (Table 3). A negative correlation was observed in each subgroup and for the whole group but the correlation is statistically significant only in the latter case and for the embryonal carcinomas.

4. *Tumour kinetics, survival and age of the patients*

A correlation between D.T. and age was observed in only one specific group, i.e. hematosarcomas. For the other subgroups, no correlation was found between survival and age of the patients.

The mean patient age varied with histological type (Table 4—Fig. 6). The patients were oldest in those groups with long D.T. and survival times (i.e. carcinomas).

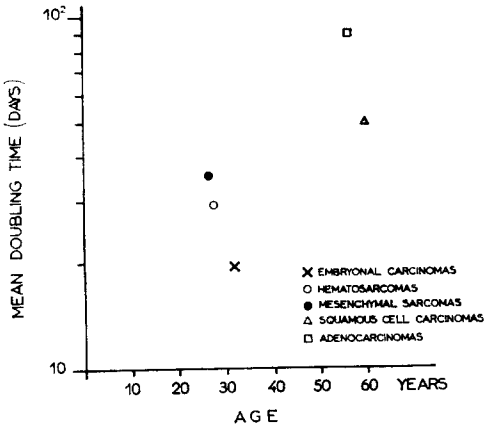


Fig. 6. Relationship between the mean D.T. and the mean age of the patients within the different pathological groups.

Considering the ensemble of all patients, the correlation between age and growth rate can be explained by the correlation between histological type and age. Children and young adults usually present with embryonal tumours, hematosarcomas and mesenchymal sarcomas, while carcinomas usually occur in adults and particularly in the aged.

DISCUSSION

1. The distribution of the survivals for the 203 patients with generalised cancer was found to be log-normal which is in agreement with previous observations [13]. This suggests that any large group of patients with disseminated cancer include some for whom the survival, even without treatment will be long.

The correlation between D.T. and survival is very significant ($P < 0{\cdot}01$) although the correlation coefficient is only 0·60 (Table 2). However, it is not yet possible to generalise for all human tumours since this work was carried out for pulmonary metastases. Furthermore, the sample of 203 patients used in this study was not representative of all human tumours. Only 55% (112 cases) had carcinomas whereas these represent 85% of all human tumours [14]. This discrepancy is probably due to the fact that sarcomas and embryonal carcinomas more frequently give rise to metastases which give clear X-ray pictures.

2. The importance of this bias can be limited by considering histological subgroups [7]. In any case, the relationship between D.T. and survival are similar when considering specific histological types or the ensemble. In addition, the differences in the correlation coefficients for separate groups and the entire sample are not very great.

Several factors could have reduced the correlation between D.T. and survival. The measure-

Table 4. Correlation between: 1. *Log D.T. of the tumour and age of the patient.*
2. *Age of the patient and log. survival time (delay between diagnosis of the primary tumour and death)*

Pathological groups	Correlation coefficient between age of the patients and D.T. of lung metastasis	Correlation coefficient between age of the patients and survival time	Age of the patients (mean value)
Embryonal tum.	0·30 (N.S.)	−0·09 (N.S.)	31·6 years
Hematosarcomas	0·58 (0·05)	0·27 (N.S.)	27·4
Mesenchymal sarcomas (fibrosarc. excl.)	0·12 (N.S.)	0·05 (N.S.)	26·4
Squamous cell carcinomas	0·08 (N.S.)	−0·18 (N.S.)	59·9
Adenocarcinomas	−0·07 (N.S.)	−0·07 (N.S.)	56·0
Fibrosarcomas	0·19 (N.S.)	−0·28 (N.S.)	41·9

ment of the growth rate of human tumours is not very accurate [6]. The delay between the diagnosis of the primary tumour and death, which has been called here survival, only includes the course of the tumour after diagnosis, that is when the tumour was already sufficiently big to be detectable. This excludes the time interval, which is probably considerable, for the tumour to reach the size at diagnosis. However, even if the number of cancer cells differs for each patient at the moment of diagnosis, these differences are not sufficient to explain the variations in survival time. In addition, there is no reason why there should be systematic differences in the size of tumour at diagnosis for the various histological types. Finally, the data consists of only one growth rate per patient, that of pulmonary metastasis. But it is known that pulmonary metastases do not have the same growth rate as primary tumours [7]. Furthermore, it is far from certain that, for the same tumour, the growth rate will remain constant for the whole period [12, 6]. The relative errors introduced by these 2 last factors are reduced since it was the D.T. of the lung metastases that was measured for all patients.

3. While it may not be surprising to observe that the survival times of patients increase as the D.T. of the tumours increase, it is more interesting to observe that what we have called the multiplication coefficient decreases as the D.T. increases. This correlation is statistically significant for the group as a whole and for the embryonal carcinomas. For the adenocarcinomas, the correlation is very low. For the other histological types, this correlation is not significant, though of the same magnitude and in the same direction.

The validity of a correlation between a quantity and another quantity which is a quotient of the first one is exposed to a bias known as spurious correlation. The probability of this bias is great when the precision of the measurement is low. Even if the precision of each D.T. is low, the mean values for each histological group is more accurately known. The fact that when comparing mean values of the D.T. and of the multiplication coefficient for the five histological groups, one finds the same tendency toward high values for the multiplication coefficient for groups with short D.T.s supports this finding.

If the multiplication coefficients were the same for both adenocarcinomas and embryonal tumours, then the average survival time of patients with adenocarcinomas should be twice as long as observed. For an equal D.T., different multiplication coefficients (and therefore different survival times) were found for different histological types. This observation shows that the survival of cancer patients is function not only of growth rate but also of the histological nature of the tumour. This leads one to pose the general problem of the mechanism of death of cancer patients.

The classical hypothesis, generally accepted either explicitly or implicitly, is that death is related to the presence of a critical number of tumour cells. This is in fact observed in certain experimental tumours [1, 2]. If the assumption is made that the growth rate remains constant for the duration of a tumour, then it is possible to estimate this critical number of cells at the moment of death (Fig. 7). It varies considerably with histological type. For instance, the average number of D.T. between diagnosis and death is 6·5 for adenocarcinomas and 13·3 for embryonal tumours. Even if the tumour sizes were very different at presentation, this cannot explain such a large discrepancy (6·8 doublings is a factor of about 100). The critical cell number therefore appears to be smaller for those tumours which grow more slowly. This is the opposite of what would be expected, a priori, since it seems reasonable to hypothesize that an organism would have in some way more time to adapt to a slowly growing tumour.

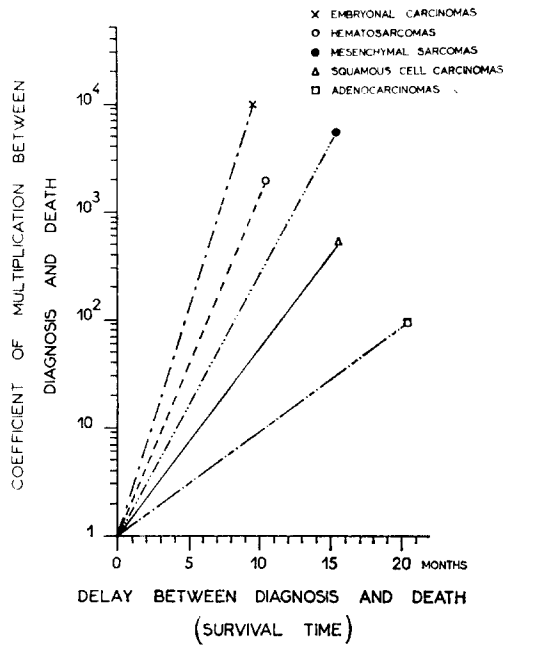


Fig. 7. Relationship between the coefficient of multiplication and the delay between diagnosis and death within the different pathological groups.

One possible objection is that the method used to determine the critical number is invalid. It is likely that the growth of pulmonary metastases is accompanied by considerable cell

loss [15]. If the concept of cell loss has enabled a better understanding of the kinetics of proliferation of animal and human tumours, it does not permit the separation of lost cells into 2 groups: those which migrate and those which die. If the lost cells form distant colonies, then the critical number will have been underestimated. The 2 extreme histological groups may again be considered as examples. Embryonal tumours have a mean survival time of 9·5 months corresponding to 13·3 D.T.s, adenocarcinomas have a survival time of 20·4 months which corresponds to only 6·5 D.T.s. It would be necessary to suppose that the actual cell number D.T. was 1·5 month rather than 3 months, the figure indicated by measurement of lung metastases, to correct for that discrepancy. In order to achieve this new value, it would be necessary that for each cell that divides and remains in the original site, a second cell migrates and multiplies elsewhere. But it is known that only a small proportion of migrating cells succeeds in establishing themselves elsewhere [16]. Furthermore, this explanation does not agree with data on the L.I. of various types of tumours [17] and with values for the cell loss in relation to the histological type of the tumours [17].

4. Since in human tumours, death does not look related to the presence of a fixed critical number of tumour cells, it would be interesting to find a correlation with another parameter.

We calculated, on the basis of data published in another paper [17] (Table 5) for each histological subgroup, the time integral of 5 different equations corresponding to:

the total number of tumour cell—days
the total number of cycling cell—days
the total number of quiescent cell—days
the total number of cell—births
the total number of cell—deaths.

For none of these 5 integrals, a similar value was found in the various histological groups. It seems therefore that death is related neither to the total number of cell-births or cell-deaths, nor to the total number of malignant cell-days as it would be the case if each malignant cell would secrete each day some toxic compound. The smallest difference between the extreme values in the various histological groups is observed for the time integral of the number of quiescent tumour cells. The meaning, if one, of this observation deserves further study.

5. The preceeding discussion which showed that the critical number of tumour cells tended to be

Table 5

Histological type	Survival (days)	D.T.	Cells present × surv. (A)	Cells produced × surv. (B)	Cells lost × surv. (C)	Cycling cells × surv. (D)	G^0 cells × surv. (E)
Embryonal tumours	285	19·5	70	1170	1100	63	7
Hematosarc.	315	29·5	7	116	109	6	1
Mesench. sarc.	465	35·6	44	151	107	7	37
Squamous cell carcinomas	468	51	4	43	39	1	3
Adenocarcin.	612	90	1	5	4	0·08	1

$$\int_0^S N_A\,dT = \frac{N_0}{K}(e^{KS}-1)\ (K = LN\,2/D.T.)\ (S = \text{survival time})$$

$$\int_0^S N_B\,dT = \frac{N_0}{K(1-\phi)}(e^{KS}-1)\ (\phi = \text{cell loss})$$

$$\int_0^S N_C\,dT = \frac{N_0 \cdot \phi}{K(1-\phi)}(e^{KS}-1)$$

$$\int_0^S N_D\,dT = \frac{N_0 \cdot GF}{K}(e^{KS}-1)$$

$$\int_0^S N_E\,dT = \frac{N_0(1-GF)}{K}(e^{KS}-1)\ (GF = \text{growth fraction}).$$

All the results must be multiplied by 10^{10}.

a function of growth rate does not take into consideration the possibility of the variation of growth rate with respect to time. If a more complex model, for example, the Gompertzian model, is used for tumour growth, then the observed facts could be accounted for by an alternative hypothesis, that the faster the tumour is growing, the sooner its growth rate will start to decrease. Some observations have been made showing a slowing up of the growth rate of certain human tumours [15]. It is at present impossible to tell whether this occurs preferentially in an initially fast growing cancer or whether there is a correlation with histological type. At present, there is no supporting evidence for this hypothesis and it is unlikely that any such effects could be sufficient to account for the observed discrepancies.

6. Data from experimental tumours show that, in small rodents, the critical size of the tumour is relatively much greater than in man and that similarily the growth rate is much faster [4, 16]. This observation is then in general agreement with the suggestion that the critical size of the tumour will be greater if the growth rate is more rapid.

A review published in 1953 [18] provides data on the histological type of the tumour and on the survival of the mice after implant for 189 transplanted tumours (75 adenocarcinomas, 58 mesenchymal carcinomas, 56 hematosarcomas). We calculated the mean and the median values of the survival for each of the 3 histological groups (Table 6).

These values differ significantly. Furthermore, there is a significant correlation between the human data found in this paper and the experimental values ($P \times 0{\cdot}01$). In both cases, the survival is shortest in hematosarcomas and longest in adenocarcinomas (Fig. 8).

Human primary tumours were treated and patients died from multiple metastases; on the other hand, animals were not treated and in most cases died without or with few metastases. In spite of these differences, the histological types exert in both cases a similar pre-eminent influence on survival. These findings underline furthermore that experimental tumours may provide a model for the analysis of the cause of death.

7. Fibrosarcomas present special problems since it is for this group that the correlation is the smallest. However, the dispersion of survival is wide. It is possible that several tumours, benign at diagnosis but malignant at a later stage, have been included in this group. This would explain some surprisingly large values for survival that have been observed.

Table 6. *Delay between graft and death for* 189 *different experimental tumours*

Histological type	Number	Geometric mean value of the survival	Median value of the survival (probit-log)
Hematosarcomas	56	22·8 days (19·0–27·4)	21·8 (19·2–25·9)
Mesenchymal sarcomas	58	34·0 (29·3–39·3)	32·8 (28·9–37·1)
Adenocarcinomas	75	45·3 (38·7–53·0)	44·2 (37·9–51·5)

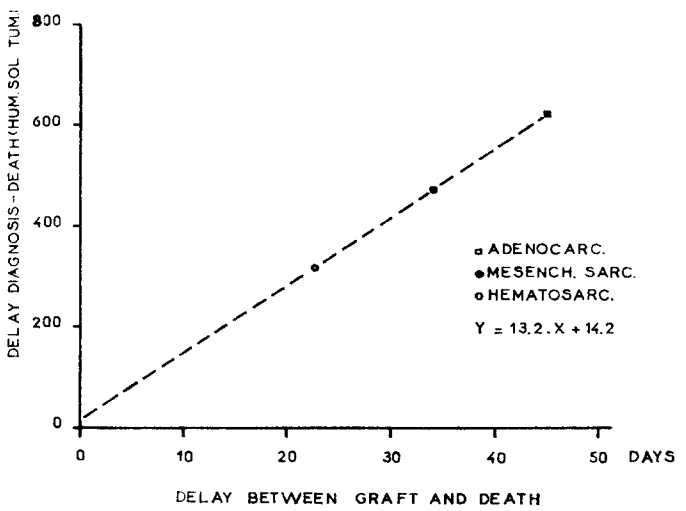


Fig. 8. *Relationship between delay diagnosis–death (human tumours) and delay graft–death (experimental tumours).*

REFERENCES

1. H. E. SKIPPER, F. M. SCHABEL, JR. and W. S. WILCOX, Experimental evaluation of potential anticancer agents. XIII. On the criteria and kinetics associated with curability of experimental leukaemia. *Cancer Chemother. Rep.* **35,** 1 (1964).
2. Y. MARUYAMA and W. BROWN, The growth of murine lymphomatous tumour-cells as determined by host survival-time. *Int. J. Rad. Biol.* **8,** 59 (1964).
3. C. CHOQUET, N. CHAVAUDRA and E. P. MALAISE, The influence of allogeneic inhibition and tumour age on the kinetics of L 1210 leukaemia *in vivo*. *Europ. J. Cancer* **6,** 373 (1970).
4. H. A. VAN PEPERZEEL, *Patterns of Tumour Growth after Irradiation* (Thesis). Edited by DRUKKERIJ VAN DENDEREN, Gröningen (1970).
5. G. G. STEEL, Cell loss from experimental tumours. *Cell Tiss. Kinetics* **3,** 193 (1968).
6. E. P. MALAISE, Cinétique des tumeurs humaines in "La cinétique de prolifération cellulaire" 375 (1971). Institut National de la Santé et de la Recherche Médicale. Paris.
7. A. CHARBIT, E. P. MALAISE and M. TUBIANA, Relation between the pathological nature and the growth rate of human tumors. *Europ. J. Cancer* **7,** 307 (1971).
8. V. P. COLLINS, R. K. LOEFFLER and H. TIVEY, Observations on growth rates of human tumours. *Amer. J. Roentgenol.* **7,** 988 (1956).
9. V. P. COLLINS, Time of occurence of pulmonary metastases from carcinoma of the colon and rectum. *Cancer (Philad.)* **15,** 387 (1962).
10. K. BREUR, Growth rate and radiosensitivity of human tumours. *Europ. J. Cancer* **2,** 158 (1966).
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,** 863 (1968).
12. P. RAMBERT, E. P. MALAISE, A. LAUGIER, M. SCHLIENGER et M. TUBIANA, Données sur la vitesse de croissance des tumeurs humaines. *Bull. Cancer* **55,** 323 (1968).
13. J. W. BOAG, Maximum likelihood estimates of the proportion of patients cured by cancer therapy. *J. roy. Stat. Soc.* **11,** 15 (1949).
14. CONNECTICUT STATE DEPARTMENT OF HEALTH, *Cancer in Connecticut* (1965). Hartford, Connecticut (1970).
15. G. G. STEEL, Cell loss as a factor in the growth rate of human tumours. *Europ. J. Cancer,* **3,** 381 (1967).
16. E. FRINDEL, F. VASSORT, E. P. MALAISE et M. TUBIANA. La cinétique de prolifération cellulaire des tumeurs humaines *in vivo* et *in vitro*. *Bull. Cancer* **55,** 51 (1968).
17. E. P. MALAISE, N. CHAVAUDRA and M. TUBIANA, The relationship between growth rate, labelling index and histological type of human solid tumours. *Europ. J. Cancer* **9,** 305 (1973).
18. L. J. DUNHAM and H. L. STEWART, A survey of transplantable and transmissible animal tumors. *J. nat. Cancer Inst.* **13,** 1299 (1953).

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Behaviour of Wilms Tumour and Normal Metanephros in Organ Culture

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Abstract—*Forty nephroblastomas and ten normal metanephros from 3–4 month human foetuses were maintained in organ culture for 3–7 weeks. Their progress was studied by light and electron microscopy. The initial state of differentiation influences the evolution of the nephroblastoma tissue in culture; the epithelial elements survive the longest, and tumours from younger children had the best viability in culture.*

NEPHROBLASTOMA or Wilms' tumour, is a malignant neoplasm of the kidney found most frequently in children under the age of ten. The histology, although exhibiting variation from one tumour to another, has the appearance of embryonic metanephric tissue [1, 2]. This morphological resemblance to the developing kidney is the basis of the generally accepted view that the tumour is of embryonic origin.

As a first step in a study of the differentiation in nephroblastoma, the behaviour of 40 tumours grown in organ culture has been studied. Their structure has been compared to the developing human kidney. Ten of these tumours have also been studied by electron microscopy. This has enabled us to observe their cellular evolution *in vitro*.

MATERIAL AND METHOD

Ten metanephros from 3 to 4 month human foetuses were used as controls. The tumour tissue was derived from 40 Wilms' tumours from 18 girls and 22 boys, all less than 7 years old; 34 were primaries and 6 recurrences. The recurrent tumours had all been irradiated before surgery.

The cultures were set up as soon as possible after surgery. Five explants of each specimen, measuring about 1 mm^3, were mounted on lens paper, or millipore filter, covering the grid of a Falcon plastic organ culture chamber. The culture medium was Eagle's medium (Pasteur Institute) containing 10% de-complemented calf serum, 200 u/ml penicillin, 0·5 mg/ml streptomycin and 1 mcg/ml fungizon. The medium was renewed twice a week. The cultures were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 and maintained for 3–7 weeks. Samples were examined weekly by light microscopy.

Tritiated thymidine (1 μCi/ml, S.A. 20 μCi/mM) was added for the last 5 hr of the culture period to some of the organ cultures.

Light microscopy

Explants were fixed for 3 hr in Bouin alcohol, embedded in paraffin, cut at 5 μm and stained with haemalum eosin, PAS or Masson trichrome.

Autoradiography

After hydration, the slides were coated with Ilford K2 film, exposed for 8 days at 4°C and then developed in Kodak 19B, fixed in Radio AL4 and lightly stained with haemalum.

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Electron microscopy

The explants were fixed for 2 hr at pH 7·4 in 3% glutaraldehyde in phosphate buffer, then washed with the buffer and post-fixed with 1% osmium tetroxide for 2 hr. The specimens were embedded in Epon, 900 Å sections were cut and stained with uranyl acetate–lead citrated by Reynolds' method [3] and examined in a Philips EM 200 electron microscope.

RESULTS

A. *Normal embryonic tissue*

The explants were taken from the kidney cortex. In foetuses of 3–4 months old the cortex is a peripheral blastematous area from which the nephrons will develop.

During culture the size of the explant did not increase. Epithelial structures proliferated and produced an encapsulation. Necrosis occurred at various stages of the cultures, either in the middle of large explants or selectively in certain histological structures. The results varied according to the duration of the culture and to the degree of differentiation in the explants.

Explants from the immature part of the cortex retained their initial histological appearances for 3 weeks in culture without necrosis. However, the few existing differentiated tubules or glomerular precursors present some signs of proliferation, mitosis, encapsulation and sometimes dilatation.

Explants from the differentiated part of the cortex presented some histological changes from the first week (Fig. 1); encapsulation and tubular dilatation, as well as the beginning of cellular necrosis in the periphery of glomeruli and stromal fibrosis. These degenerative processes become progressively more marked as the culture proceeded and after 3 weeks the glomerular necrosis was complete. The lumen of the tubules were filled with necrotic debris and the fibrosis appeared to surround the epithelial structures. However, the epithelial structures survive and sometimes proliferate (encapsulation, mitosis in the tubular epithelium).

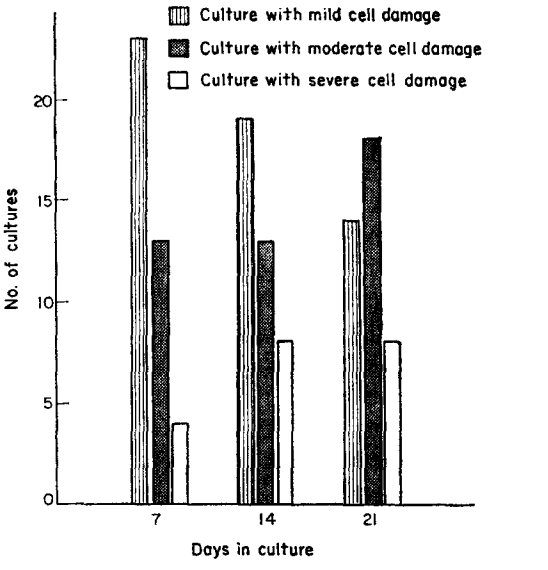
B. *Tumour samples*

The various common forms of Wilms' tumour were represented in the 40 tumours examined in this series. Only two tumours were completely undifferentiated; in one the histological appearance was that of non-organised blastaema tissue and in the other the appearance was of lobulated tissue with necrotic changes.

In 9 tumours a few differentiated tubules in a mesenchymatous type of basic stroma were observed. This stroma was relatively vascularized, sometimes having the appearance of connective tissue. Cystic formations were observed in two cases. In addition to these differentiated states, 11 tumours also showed glomerular precursors. On the whole these tumours contained more epithelial cells than other tumors.

Striated, or smooth muscle differentiation, was observed in 18 cases, the cells were dispersed or grouped together in the basic stroma; among these 18 tumours, 6 showed tubular and 9 had glomerular differentiation.

Table 1. *Viability in relation to duration of culture*



Comparison of irradiated and non-irradiated tumours shows that irradiation appears to cause necrotic changes often visible macroscopically together with a tendency to induce muscle differentiation in the tumour (see Table 2).

In vitro *evolution*

As in the normal foetal kidney, there is no increase in the size of tumour explants, central necrosis or death of some of the histological structures was observed as well as encapsulation when epithelial structures were present. In two cases the cells grew outwards on to the support (Millipore, or lens paper). Multinucleate cells were found close to the explant. The evolution of the explants based on the histological

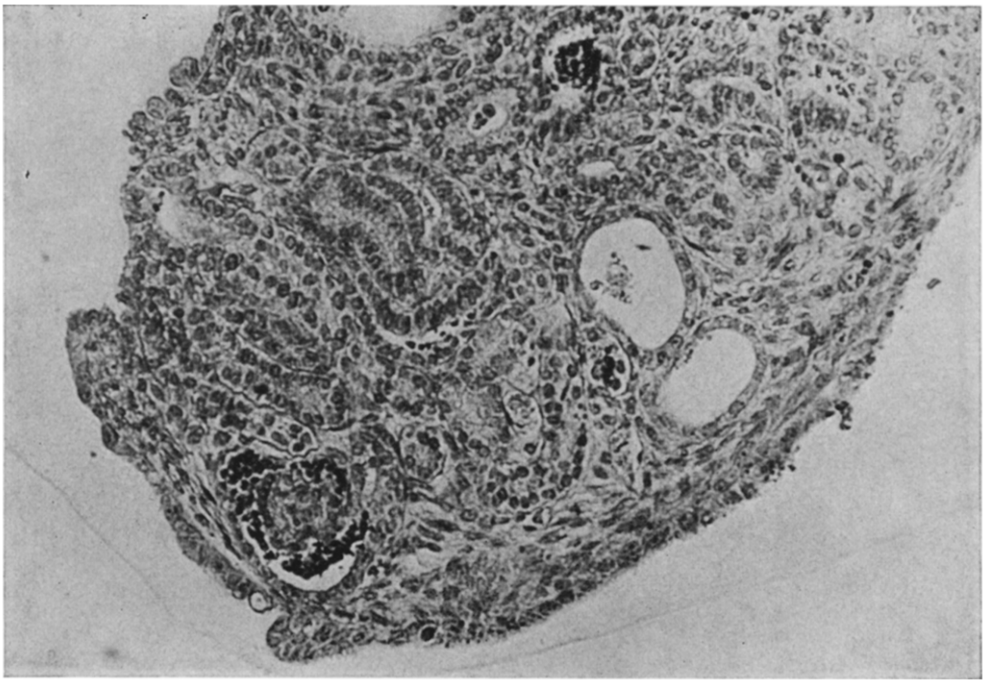


Fig. 1. *Metanephros from* 3 *months human foetus after one week in culture. Note: explant encapsulation early necrosis in the glomeruli, tubular dilatation and well preserved epithelial strucutres* (*HE* × 126).

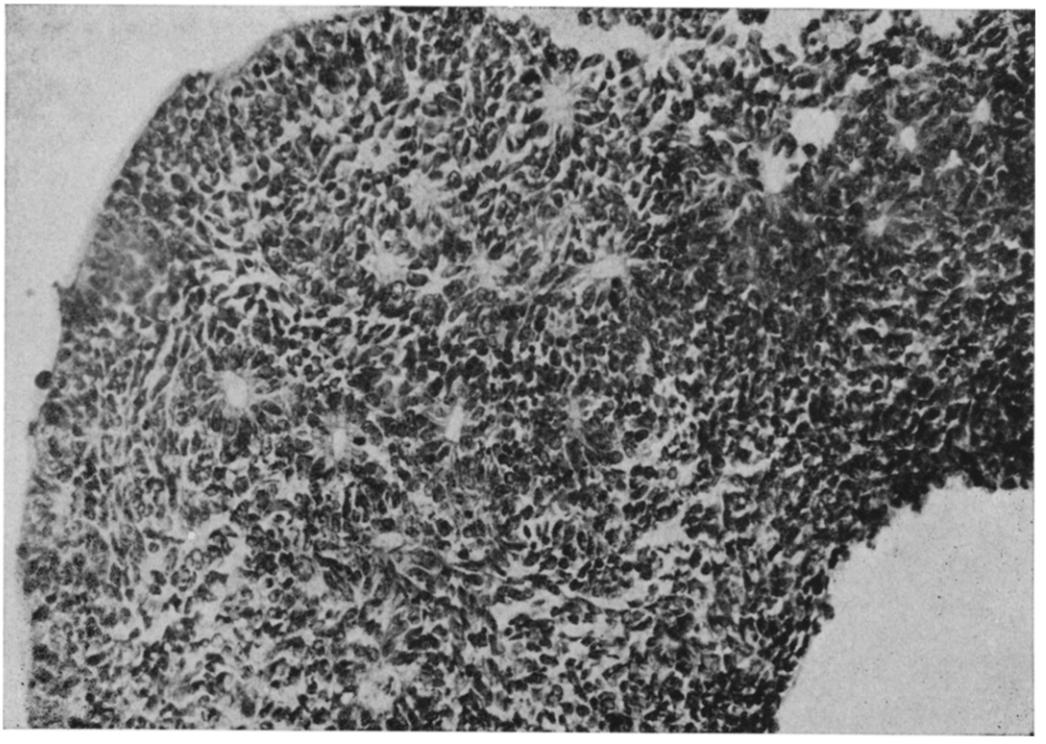


Fig. 2. *A metastatic nephroblastoma in the liver. Histological appearance after* 3 *weeks in culture* (*HE* × 126).

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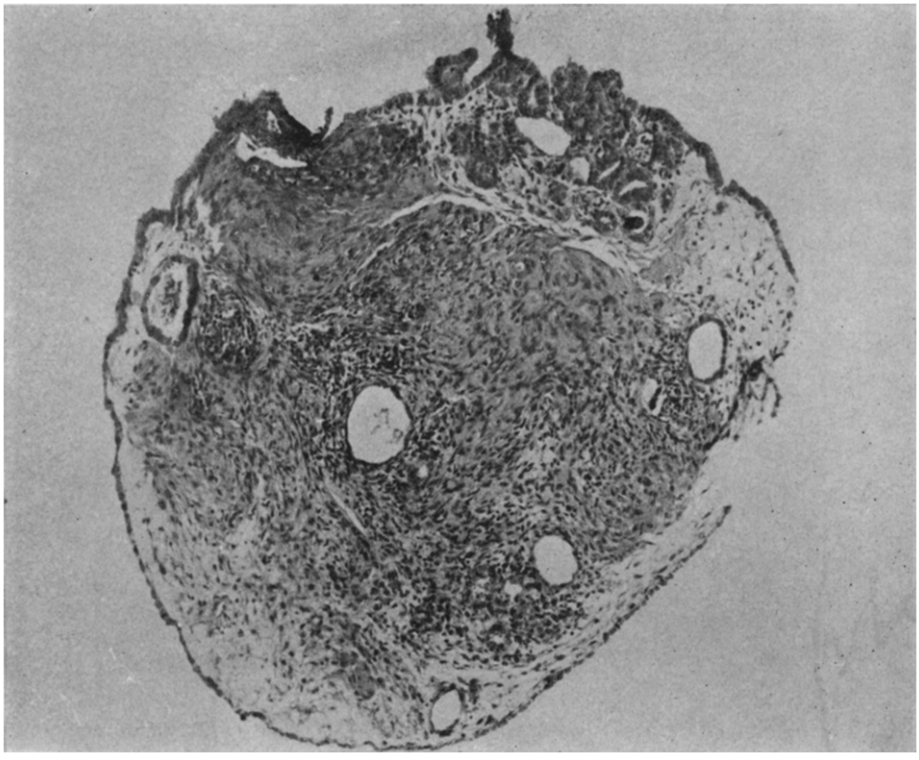


Fig. 3. Differentiated nephroblastoma after 2 weeks in culture. Explant encapsulation, tubular dilatation, early fibrosis (HE × 48).

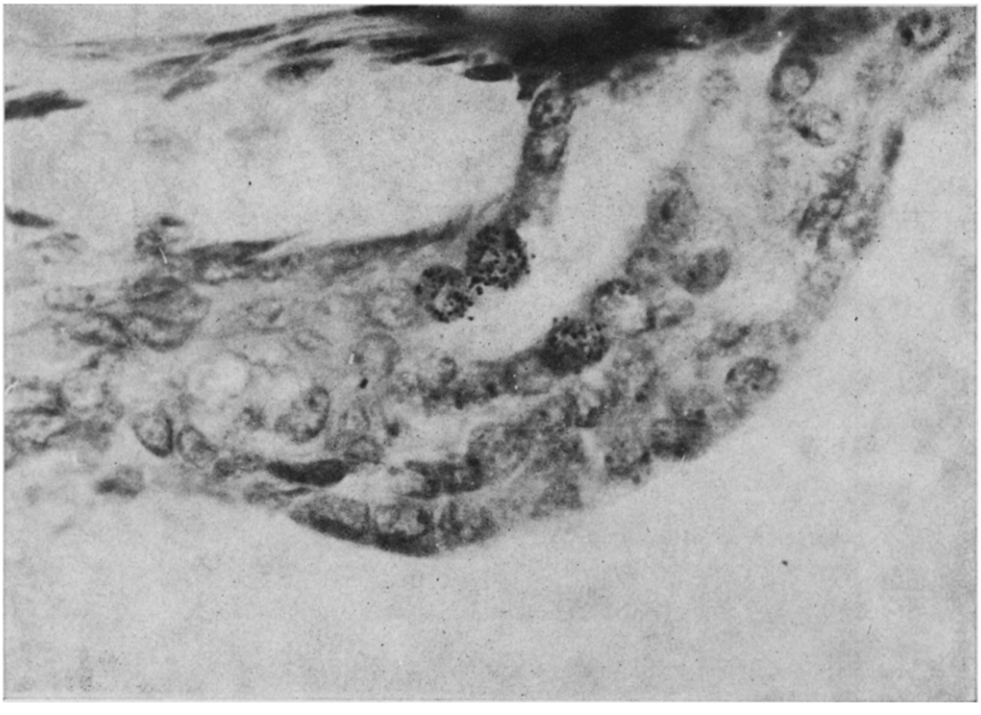


Fig. 4. ^{3}H–TdR incorporation in the tubular cells after 32 days in culture (HE × 800).

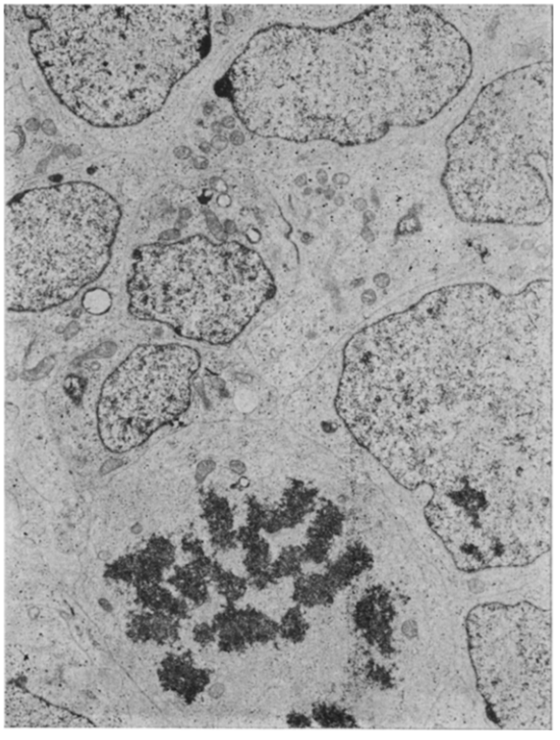


Fig. 5. *Microphotograph of a well preserved cell mass containing a mitotic figure. Desmosomes and and some degenerating lipidic vacuoles are present.* (*Seven days in culture* × 18,500. *Uranyl lead acetate*).

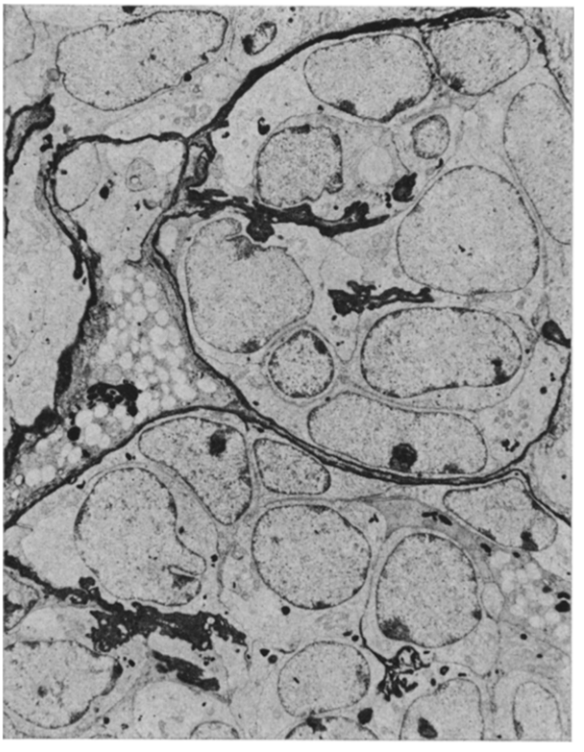


Fig. 7. *Preglomerular structure which shows a membrane differentiating between and around the cell masses. Note the presence of one lipophage.* (*Seven days in culture* × 9500 *silver impregnation*).

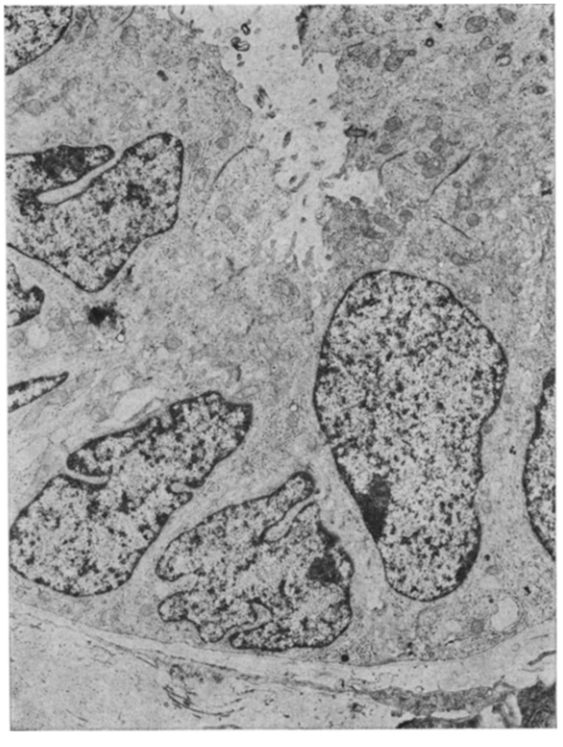


Fig. 6. *Well preserved tubular section with basement membrane and some β cyto-membrane formations. The apical zone shows some short microvilli and several ciliary formations.* (*Seven days in culture* × 18,000. *Uranyl lead acetate*).

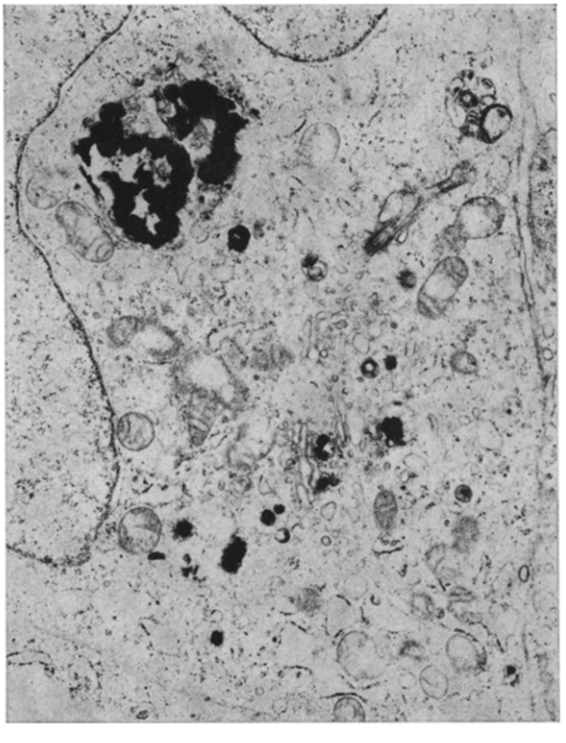


Fig. 8. *Higher-power view, illustrating the beginning of lipidic degeneration. Vacuoles, containing necrotic electron-dense material and myelin figures, cell organelles* (*mitochondria, Golgi, ergastoplasm*) *are to be seen, as well as a ciliary formation, part of the nucleus and its membrane.* (*Seven days in culture* × 40,000. *Uranyl lead acetate impregnation*).

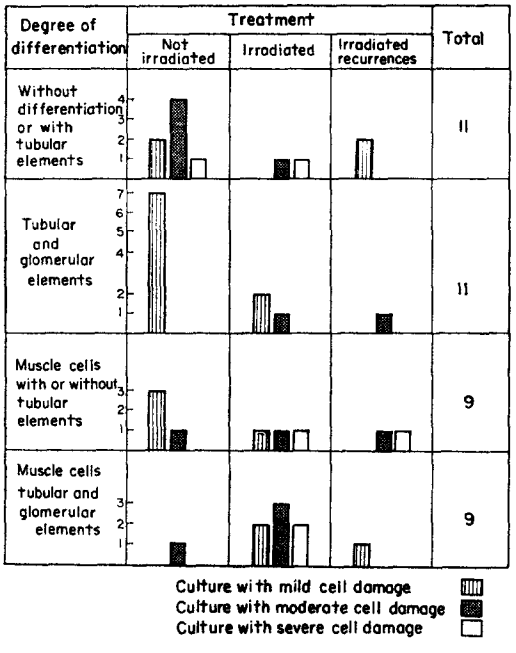
examination and necrosis were classified as mild, moderate and severe damage.

The changes varied according to the duration of the culture (Table 1). Sixty per cent of the explants showed a good survival at 7 days and 35% at 21 days. Three factors influenced the results of the cultures: the condition of the tissue when removed, the histological type, and the patient's age.

(*a*) *Effect of irradiation* (Table 2)

Two thirds of the 19 non-irradiated nephroblastomas had a good survival after 3 weeks as explants. This was reduced to one third in the 15 irradiated nephroblastomas. Irradiated tumours showed necrotic changes that decreased the explants survival. The basic stroma was generally affected by a fibrous degeneration after one week in culture. However, when epithelial components were present in the original tumour they usually remained intact and proliferated *in vitro*.

Table 2. Behaviour of explants in relation to treatment and differentiation of the tumour in vivo



The 6 cases of recurrent nephroblastoma showed a pattern of proliferation and survival in culture intermediate between the unirradiated and the irradiated tumours.

(*b*) *The influence of histological type* (Table 2)

The mesenchymal and epithelial components, present in the tumours in a more or less differentiated state, survived in various ways *in vitro* and affected the results of culture.

The explants were of 4 histological types:

(1) undifferentiated nephroblastomas, and tubular differentiated nephroblastomas

(2) differentiated nephroblastomas with tubular and preglomerular formations

(3) differentiated nephroblastomas with muscular differentiation (with or without tubular formation)

(4) differentiated nephroblastomas with muscular differentiation with tubular and glomerular formations.

Undifferentiated nephroblastomas and tubular differentiated nephroblastomas. Explants of these tumours grew well. The mitotic level was generally high and maintained *in vitro*. Two series of cultures from hepatic metastases of nephroblastomas were kept up to 38 days without histological modification of the explants (Fig. 2). During the experiment the explants flattened out on the lens paper, but cell arrangements, incipient tubular formations and numerous mitotic figures remained unchanged. Cell necrosis was limited and dispersed and did not seem to increase with time.

As soon as connective tissue predominated over epithelial cells, the explant very rapidly took on a fibrous aspect and cell necrosis appeared after one week. Cell limits became blurred, chromatin faded and nucleoli increases in size.

Nephroblastomas with tubular and glomerular differentiation. The neoplastic blastaema component was particularly well represented in this group of tumours, which explains the positive results given in Table 2.

The most differentiated nephroblastomas comparable to the developing kidney were evolving in the same way *in vitro*: mitosis, encapsulation, tubular dilatation. Most explants maintained this modified but non-necrosed aspect for several weeks, and ^{3}H–TdR incorporation was demonstrated in the tubular cells in a 32 day culture (Fig. 4). Progressive accumulation of necrotic matter in the lumen of undilated tubes, rapid necrosis of the glomerular grouping and a tendency to fibrous degeneration in the basic stroma were noted in some well differentiated forms (Fig. 3).

Muscular differentiation was not maintained *in vitro*: muscular type cells became round, their outlines blurred and the nuclei showed pykno-

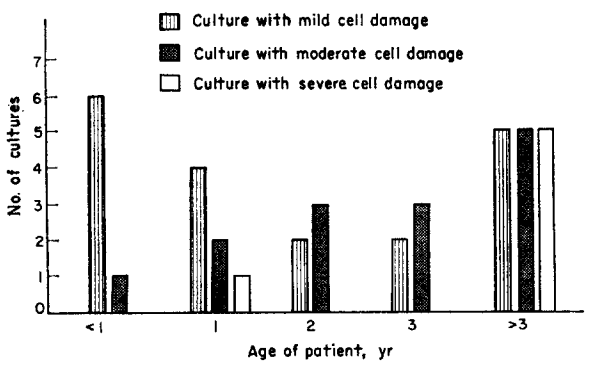
sis after one week. Cultures persisted due to the presence of epithelial or slightly differentiated mesenchymatous components.

Vascularization. The small vessels could not always be observed, but on the whole, although red blood cells disappeared quite rapidly, the endothelial cells seemed to persist, at least for one week in culture.

(*c*) *Importance of the child's age* (Table 3)

The best culture results were obtained with tumours from children under the age of 2 years: more than two thirds of the results were positive, as against one third after this age.

Table 3. Patient's age and explant survival in culture



The fact that, in our hospital, irradiation was rarely performed on children under the age of 2, affects this finding only to some extent. No relationship could be demonstrated between histology of the tumour and the patient's age.

ELECTRON MICROSCOPIC APPEARANCES

Since a comparison of the ultrastructure of Wilms' tumour to normal developing metanephros has been reported previously [4], this description will be restricted to nephroblastoma. The ultrastructural aspect of these 10 nephroblastomas before culture will be described elsewhere [5].

Briefly, it is a collection of cells presenting different cytoplasmic aspects, either dispersed in the basic stroma or grouped in more or less structured clusters. Isolated cells and cell clusters are enclosed in a mesh of collagen fibrils, often accompanied by amorphous material that is not very dense to electrons.

It is extremely difficult to establish the kinetics of the different cell elements by electron microscopy during culture. The picture on the whole is that of a slow evolution towards cell necrosis, but is should be remarked that even before culture, there were some rare necrotic patches in the interstitium, and some myelin figures and lipid inclusions in the cells; the presence of necrotic matter in the lumen of tubular sections was also noted.

(*a*) *Stroma and isolated cells*

A distinct tendency to fibroblastic transformation of all elements of the stroma was observed during culture, accompanied by an increased amount of fibrous degeneration. Fibroblasts themselves either had numerous vacuoles invading the cytoplasm or large lipid inclusions that disorganised the cells.

The large isolated cells also presented numerous vacuoles with dense contents, myelin figures and a peculiar appearance of the cytoplasm, which was granular and blurred, with disappearance of cell organelles, suggesting progressive necrosis of the cell. Other cells were studded with vacuoles and the perinuclear space was oedamatous. Sometimes the plasma-membrane seemed to split and the cytoplasm spilled out into the interstitium.

Other cells acquired a very distinct lipophagic character, with lipid vacuoles completely occupying the cytoplasm.

At the most advanced stage of degeneration, pyknosis of the nuclei, followed by total necrosis of the cell elements were observed.

(*b*) *Cell masses*

These cells can sometimes persist for several weeks *in vitro* without very important modification (Fig. 5). However, after one week, large vauoles full of necrotic waste matter may be found in the cytoplasm. They often appear together with myelin figures contained in both cytoplasmic and lipid vacuoles. These phenomena are visible even in mitotic cells. Later, the perinuclear space is sometimes detached, indicating cell damage. The nuclei become pyknotic.

(*c*) *Tubular structures*

The cell masses that are already differentiated, and thus showing a tubular structure, seem to be less rapidly affected by necrosis. Even though these structures showed more or less widespread changes after 4 weeks in culture, they always preserve their shape and limitation by a basement membrane.

There are several types of change: appearance of vacuoles filled with necrotic matter and lipid inclusions, very pronounced dilatation

of the endoplasmic reticulum. These various signs of degeneration can coexists.

Ciliated formations observed in the cell masses and tubular sections are found to be intact, whatever the delay in culture (Fig. 6).

(d) Preglomerular structures

A pre-glomerular formation was observed after one week's culture, and after silver staining this structure was seen to possess a characteristic membrane (Fig. 7).

(e) Muscular differentiation

From the first week of culture, the muscular fibres lost their characteristics and became amorphous material, detectable in cells undergoing lysis or dispersed in the more or less fibrous basic stroma.

Summarizing these results, after one week we observed fibrosis which seemed to spread progressively in the course of time. Different cell elements observed before culture sometimes persisted in this fibrosis up to 4 weeks, but with more or less marked degenerative aspects. However, the structured cell masses and tubular formations seemed to resist necrosis for a longer time. It should be noted that whatever the duration of culture, mitosis was always observed even after 30 days, sometimes in cells with preliminary signs of degeneration.

Degeneration seems to begin with the appearance of lipid vacuoles that are observed in cell cytoplasm (Fig. 8). The nucleus becomes pycnotic later, and in the end the cell dissociated into the basic stroma.

Finally, it should be noted that the different survival and degenerative stages may coexist at any time in the culture, and that normal appearances of the different elements may be observed after long periods of culture, or necrosis may be almost complete after one week.

DISCUSSION

Although malignant tissues have been sometimes maintained in organ culture for several years with preservation of antigenic [6] and chromosomal [7] character, they are usually of short duration and are utilized as an element of comparison for the evolution of normal tissue [8, 9, 10] or for therapeutic experiments [11].

Cultures of human embryonic tumours are rare. Kalus [10] found two neuroblastomas in tissue culture had a good survival.

From the present results it appears that organ culture is compatible with the survival of the major part of cell components of nephroblastoma for several weeks *in vitro*. It is the epithelial elements in the tumour that tend to survive so that a morphological comparison of nephroblastoma with developing kidney tissue (blastematous components, incipient tubular and glomerular elements) can be made. Some of the usual reactions of most explants in short-term organ culture are observed: central necrosis, epithelial proliferation and reaction to foreign bodies [8]. Mitotic figures were often seen but no noteworthy growth of the explants was observed in proportion to the duration of culture. Necrotic results and dilatation in the ureteral branches has been reported in organ culture of kidney mouse [12, 13]. That non-necrotic process of tubular dilatation observed here, not under hyperoxic conditions, may depend on the static conditions of the organ culture.

The ultrastructural study confirms that Wilms' tumour explants will either maintain cell structures for several weeks, or undergo progressive necrosis. Survival, although accompanied by cell changes is authentic. For example, organized structures and mitosis persisted in 3 cases observed by electron microscopy after long culture periods (4 weeks and more), and a relative but circumscribed fibrous invasion took place in comparison with well-preserved cell masses.

Certain histological structures survive better than others. Whether they are pathological or normal, these elements go through the same evolution *in vitro*. On the whole, epithelial structures adjust themselves the best despite observations made with cell cultures of nephroblastoma [14]. However, a basic stroma is noted, together with cell degeneration and generalized fibrosis of connective tissue. In a nephroblastoma, an evolution towards fibrosis is accentuated in the presence of muscular cells, which necrose particularly quickly during culture.

The predominance of muscular differentiation in irradiated tumours has already been observed [15]. This kind of stimulation by irradiation does not appear to persist under the present conditions of culture. The results obtained after irradiation of chick mesonephros during culture [16] supports the idea of the fragility of the interstitial cell compartment compared with the relative resistance of epithelial structures. The observations made here with cultures of irradiated nephroblastomas agree with these findings.

In conclusion, the survival of nephroblastoma in organ culture greatly depends on the degree of differentiation, the behaviour of the most

differentiated cases is similar to that of normal developing kidney. That which corroborates their morphological resemblance. Moreover, these results show that this method of organ culture is potentially applicable to the study of nephroblastoma differentiation according to the scheme already used with other kidney tumours as a transplantable rat tumour [17] and a congenital hamartoma human tumour [18].

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REFERENCES

1. R. Gerard Marchant Les tumeurs malignes du rein chez l'enfant These de Medecine, Paris No. 138 (1953).
2. R. A. Willis, *The Borderland of Embryology and Pathology*, 2nd Edn. London. (1962).
3. E. S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208–212 (1963).
4. J. Ito and W. W. Johnson, Ultrastructure of Wilms' tumour. I—Epithelial cell *J. nat. Cancer Inst.* **42,** 77–99 (1969).
5. M. F. Rousseau and B. Nabarra, Embryonic kidney tumours. Ultrastructure of nephroblastoma (to be published) in *Virchows Arch. Abt. A Path. Anat.*
6. P. Burtin, D. Buffe, D. von Kleist, E. Wolff and Et. Wolff, Mise en evidence de l'antigène carcino-embryonnaire spécifique des cancers digestifs dans les tumeurs humaines entretenues en culture organotypique. *Int. J. Cancer* **5,** 88–95 (1970).
7. J. de Grouchy and Et. Wolff, Analyse chromosomique d'une tumeur cancereuse humaine en culture organotypique. *Europ. J. Cancer* **5,** 159–163 (1969).
8. F. F. Archer, Normal and neoplastic human tissue in organ culture. *Arch. Path.* **85,** 62–71 (1968).
9. T. Holmstrom, E. Saksela, S. Nystrom and E. Saxen, Growth behaviour of human brain tumours in matrix cultures in fresh autologous serum. *Acta path. microbiol. scand. sect. A.* **78,** 313–322
10. M. Kalus, J. J. Ghidoni and R. M. O'Neal, The growth of tumors in matrix cultures. *Cancer* **22,** 507–516 (1968).
11. M. Yarnell, E. J. Ambrose, K. Shepley and R. Tchad, Drug assays on organ cultures of biopsies from human tumors. *Brit. med. J.* **2,** 490–491 (1964).
12. J. S. Resnick, D. M. Brown and R. L. Vernier, Oxygen toxicity in fetal organ culture. I. The developing kidney. *Lab. Invest.* **28,** 437–445 (1973).
13. J. F. S. Crocker and R. L. Verneir, Fetal kidney in organ culture: abnormalities of development induced by decreased amounts of potassium. *Science (N.Y.)* **169,** 485 (1970).
14. M. F. Rousseau, Etude chromosomique de 20 tumeurs embryonnaires après culture à court terme. *Biomedecine* **19,** 275–280 (1973).
15. G. A. Bannayan, A. G. Huvos and G. J. D'Angio, Effect of irradiation on the maturation of Wilms' tumour. *Cancer* **27,** 812–818 (1971).
16. L. de Ridder, Organotypical culture of irradiated and non-irradiated chick mesonephros. *Sep. Experientia* **27,** 428–430 (1971).
17. M. L. Ellison, B. J. Ambrose and G. C. Easty, Differentiation in a transplantable rat tumour maintained in organ culture. *Exp. Cell Res.* **55,** 198–204 (1969).
18. J. F. S. Crocker and R. L. Vernier, Congenital nephroma of infancy: induction of renal structures by organ culture. *J. Pediat.* **80,** 69–73 (1972).

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Long-Term *In Vitro* Culture of Pathogenic Gross Leukemia Virus in Mouse Thymus Cells

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Abstract—*Monolayer cultures were established by explanation* in vitro *of fragments obtained from a hypertrophied thymus of a two months old C3HeB/Fe mouse inoculated at birth with passage A Gross leukemia virus (GLV). The cytomorphology of these cultures was first mixed showing fibroblasts and epithelioid cells but became more uniform in later passages of either the "wild" cell population (TGV) or the originally ephithelioid (K8E) or fibroblastic (K10F) clones. After nearly one year of culture, the rate of cell replication stabilized at the level of* 24–30 *hr doubling time. The karyotype of these cultures was close to normal with a peak at* 40 *chromosomal value with no distinct markers. Repeated electron microscope examinations showed an abundance of C-type particles budding out from the cell surface or present in the intercellular spaces. Association of the TGV or clonal cell populations with XC cells resulted in an intense formation of foci of XC degenerating polykaryocytes characteristic for presence of infectious murine leukaemia viruses. Immunofluorescence tests with anti-GLV serum were highly positive with indexes reaching, between the 42nd and the 60th passages,* 0·94 *values with practically negative controls (index* 0·20*) with anti-Rauscher leukaemia serum. Inoculation of newborn C3HeB/Fe mice with supernatants from TGV or TGV-derived cultures, performed between the 13th and the 22nd month of culture (5th to 51st passages, respectively), resulted in Gross-type lymphoid leukaemia appearing after nearly* 3 *months of latency. Once the first symptoms (splenomegaly and adenopathy) appeared the survival time was quite uniformly limited to no more than* 4 *weeks in average. When TGV cells instead of supernatants were injected into newborn mice the latency before appearance of leukaemia was slightly prolonged. Local formation in these animals of nodules which subsequently regressed was observed. This was interpreted as a capacity of the C3HeB/Fe mice to react immunologically against Gross virus-linked or virus-induced antigens carried by the TGV cells. No other pathological symptoms were seen in these mice.*

It is concluded that thymus cultures containing cells of cytologically mixed origin but obviously no typical lymphoid elements can sustain replication of Gross leukaemia virus having maintained its high pathogenic potential for periods of time exceeding at least 22 *months.*

INTRODUCTION

THE PROPAGATION of murine leukaemia viruses of the Friend–Moloney–Rauscher (FMR) group in cell cultures is relatively easy, and cell lines infected with these viruses could be obtained without much difficulty either by explantation of lymphoid tissue from leukaemic mice or by *in vitro* infection of virus-free mouse cell cultures with a virus preparation [1–3]. Pathogenic and non-pathogenic variants of FMR viruses have been studied in experiments with such infected cultures [4, 5].

On the other hand, data concerning the *in*

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vitro culture of pathogenic Gross-type murine leukaemia virus (GLV) are comparatively scarce [6, 7]; though there are in the literature numerous reports of "spontaneous" or "endogenous" virus infections in mouse cells, where the viruses have ultrastructural and antigenic characteristics of GLV but no leukaemogenic capacity [8, 9].

The purpose of the experiments reported here was to develop an *in vitro* mouse cell culture derived from tissue of an animal with Gross lymphoid leukaemia, in order to follow the evolution *in vitro* of the infective, pathogenic and antigenic characteristics of the virus population replicating in this culture.

MATERIAL AND METHODS

Virus

The original Gross passage A virus was received from Dr. Gross in 1964. It was carried by animal to animal passages of spleen extract, with intermittent storage at liquid nitrogen temperature.

Cell cultures

Cultures of leukaemic C3HeB/Fe mouse thymus were established *in vitro* as described later and designated TGV. They were maintained by routine methods in Eagle's MEM supplemented with 10% foetal calf serum, with added penicillin and streptomycin (100 units and 100 μg per ml respectively). Passages of established cultures were carried out weekly by trypsinization with 0·25% trypsin solution.

Tests for the presence of virus in the cultures

1. *Electron microscopy.* Confluent cell monolayers in culture bottles were fixed with 1·5% glutaraldehyde and post-fixed at 4°C for 1 hr in 1% osmium tetroxide in Sörensen buffer. The sections double-stained with uranyl acetate and lead citrate were prepared and observed according to the method described previously [10].

2. *Direct XC. test.* This was a modification of the test used by Rowe *et al.* [11]. The details of the technique and method of scoring the results have been given elsewhere [12].

3. *Immunofluorescence test.* The direct immunofluorescence test (IF) was carried out on suspensions of living cells following the technique described by Möller [13]. Type-specific anti-Gross serum obtained from (W/Fu × BN) F1 hybrids rats with Gross lymphoma (kindly supplied by Dr. Old of the Sloan Kettering Institute, New York, USA) was absorbed before use in the test by incubation for 1 hr at 37°C and 1 hr at 4°C with a suspension of T5 cells, chronically infected with low leukaemogenic Rauscher virus [14]. After centrifugation at $500 \times g$ for 10 min, the supernatant was taken for use. For negative controls, type-specific anti-Rauscher BALB/c mouse serum was prepared from BALB/c mice hyperimmunized with T5 cells. This serum was absorbed with TGV cells by the method described above. Sera from BALB/c mice and rats immunized with sheep red blood cells were used as additional negative controls. Fluorescein-conjugated goat sera (anti-mouse gamma-globulin and anti-rat gamma-globulin) were purchased from Hyland Laboratories, Los Angeles, California, USA.

Animal inoculation tests

For testing the leukaemogenic potential of the material from TGV cultures, mice were used of the inbred C3HeB/Fe strain, supplied by the central animal house of our Institute or bred in our laboratory. Unless otherwise stated, mice were inoculated when less than 48 hrs-old. The supernatant for inoculation was taken from cultures started with 500·000 cells in 20 ml of medium and renewed 3–4 days before collecting of the supernatant. At this moment (7th day after seeding) the density of the culture was of the order of $2{\cdot}5 \times 10^5$ cells per ml of medium. Cells for inoculation were obtained from similar cultures.

If non-concentrated supernatant was required, the liquid was centrifuged in the cold at $500 \times g$ for 10 min and material for use was taken from the top of the tube. Concentrated supernatant was prepared as described by Barski and Youn [9]. The prepared supernatant, whether concentrated or non-concentrated, was used for inoculation either immediately or after storage at -76°C. The supernatant was injected intraperitoneally, each mouse receiving 0·2 ml of non-concentrated or concentrated supernatant. Cells were inoculated sub-cutaneously.

The animals inoculated with the TGV culture supernatants or cells were observed as a rule throughout their life-span. Enlargement of the spleen and inguinal lymph nodes was checked weekly by palpation. Not infrequently the animals experienced obvious distress in breathing due to a greatly enlarged thymus. In doubtful cases, animals were sacrified and autopsied. As an additional check, samples of spleen, thymus, liver and inguinal lymph nodes were prepared for histological examination after fixation in Bouin's solution.

RESULTS

Cell cultures and general observations

Cells from a hypertrophied thymus of a leukaemic C3HeB/Fe mouse were put into culture in July 1971. The diseased animal had been inoculated at birth two months earlier with passage A Gross virus.

To start the culture, the thymus was cut finely with scissors and after washing with PBS, small fragments of tissue were transferred to a bottle containing culture medium. At first, the growth was very slow. Only after about 7 months the first passage by mechanical scraping could be made, and in February 1972 passages by trypsinization were started.

In the early stages of *in vitro* culture the cell population seemed to contain different morphological cell types, which, broadly speaking, could be classified into: rather epithelioid cells; or, cells with a more fibroblastic appearance. If the cell monolayer was washed by gentle pipetting with culture medium, or treated for a short time with trypsin solution, the resulting suspension tended to contain mostly epithelioid cells. The remaining cells, which appeared to be predominantly of the fibroblastic type, adhered much more firmly to the glass wall of the culture vessel. However, it was impossible to separate clearly distinct cell-lines by such a crude method. Soon after the commencement of passages by trypsinization, a monodispersed cell suspension was obtained and seeded in Petri dishes. Approximately one week after seeding, small well-separated colonies were transferred separately into flattened tubes and later into culture bottles. Two such cultures obtained from the TGV line, designated K8E (epithelioid) and K10F (fibroblastic), were chosen for further study along with the wild TGV line. It must be admitted that, as time went by, the differences between K8E and K10F became less obvious given the cytomorphological variability within each of these lines.

Meanwhile, general observations on the TGV parental line were continued. Even after a number of passages, some cytological polymorphism persisted as seen in Fig. 1 (TGV cells at the 34th *in vitro* passage). The presence of occasional "giant" cells with large nuclei was typical. The plating efficiency of the TGV line was found to average 15% at the 32nd *in vitro* passage and some differences in the morphology of the colonies formed were observed. The cell doubling time was found to be 24–30 hr.

Chromosomal studies, for which Giemsa-stained preparations were used, were carried out at the 42nd and 58th passages of the TGV line *in vitro*. Karyological analysis showed, for both passages, a sharp peak at the 40 chromosomes euploid value with a secondary slight peak at the tetraploid value of 80. No easily recognisable and regularly-occurring marker chromosomes were observed.

Electron microscopy studies on TGV

Repeated observations showed the presence of C–type virus particles with some budding in early and late passages of the TGV cells.

Direct XC test with TGV and its derivatives

Numerous tests of association of XC and TGV cells *in vitro* were performed between the 30th and the 60th passages of the TGV line or its derivatives. All gave positive results at the level of ++++ according to the score proposed previously [12]. Figure 2 shows a group of polykaryocytes in a test made during February 1973, 2 days after 10^5 TGV cells (47th passage) were added to the XC cells. Tests with 10^4 TGV cells per tube were also clearly positive. The two TGV derivatives, K8E (tested at the 13th *in vitro* passage) and K10F (tested at the 10th passage) both gave in the XC test equally positive results with 10^4 cells per tube.

Immunofluorescence tests with TGV cells

TGV cells gave a regularly positive indication of the presence of Gross-type virus-induced antigen at the cell surface, as evidenced by the proportion of cells showing specific surface fluorescence in the indirect IF test. In a test made on TGV cells at the 42nd *in vitro* passage, the fluorescence index was found to be 0·72 with anti-Gross serum, as compared with very low and non significant indexes of only 0·20 with anti-Rauscher and 0·02 with mouse anti-sheep red blood cell sera. When another test was made at the 60th *in vitro* passage, the fluorescence index was 0·94 in the presence of anti-Gross serum as compared with the 0·04 index obtained parallely in controls with mouse anti-sheep red blood cell sera.

Leukaemogenicity of TGV and its derivatives

Table 1 shows the results of 7 experiments where 0·2 ml of non-concentrated, and in one case, 30 × concentrated cell culture supernatant of TGV or of its derivatives K8E and K10F was inoculated intraperitoneally into newborn C3HeB/Fe mice.

It is evident that the cultures of the TGV cell line or its derivatives, K8E and K10F,

Table 1. Results of experiments in which cell culture supernatants of TGV, or of its Derivatives K8E and K10F, were inoculated into newborn C3HeB/Fe mice.

Expt. No.	Time after establishment of TGV, K8 and K10 cultures (months)	Supernatant injected: Strain	Supernatant injected: Passage No.	Leukaemic mice: No.	Leukaemic mice: %	Average latency periods (days)	Mean survival time (days) after first symptom appeared
1A	8	TGV	2*	9/10†	90		‡
1B	8	TGV	2*	12/18†	67		
2	13	K8E	5	28/28	100	88	23
3	14	K10F	3	19/19	100	90	25
4	15	K8E	9	19/19	100	88	25
5	15	K10F	6	19/19	100	92	27
6	18	TGV	37	19/19	100	77	21
7	22	TGV	51	10/10	100	89	24

*Supernatant 30 × concentrated (in other cases, supernatant unconcentrated).

†At 90 days after inoculation (in other cases, animals were observed for at least 150 days after inoculation).

‡Not calculated, because of incomplete observation period.

produced highly leukaemogenic virus for at least 22 months after the original establishment of the TGV line *in vitro*.

A figure of less than 100% leukaemic animals in experiments 1A and 1B is questionable and most certainly underestimated, since the observations in these two experiments were not continued after the 90th day following virus inoculation whereas in other experiments, they extended over a period of 150 days.

When the observations were continued over this period, as was done in Experiments 2–7, it appeared that the latency period between the day of virus inoculation and the first symptoms of leukaemia varied chiefly between 60 and 115 days with occasional extreme cases of very early (e.g. 43 days) and very delayed (e.g. 151 days) appearance of the disease. The averages calculated for different experiments and shown in Table 1 were fairly uniform, varying between 77 days, (Exp. 6), and 92 days, (Exp. 5). The uniformity of leukaemogenic potential observed with material from different passages of TGV and derived lines was additionally illustrated by the small variation in mean survival time of the inoculated animals, after the appearance of first symptoms of leukemia which varied between 21 and 27 days for all the experiments. The survival time was not related to the latency period, and once an animal showed symptoms of leukaemia, the progress of the disease was consistently quite rapid.

From the data available, we did not feel able to conclude that the virus material harvested in K8E and K10F cultures differed from each other or from that of the parent TGV cell culture as regards the observed latency period or survival time after the appearance of first noticeable symptoms of leukaemia.

Tumorigenicity of TGV cells

When cells of TGV at the 2nd *in vitr* passage were inoculated (10^6 cells by subcutaneous route) to 2 litters of newborns and to their mothers (5×10^6 cells in the same way), no tumours were observed in the total of 13 inoculated animals. However, 2 of the mice inoculated as newborns showed, not unexpectedly, enlargement of the spleen 70 days after inoculation, and eventually died of leukemia.

In later experiments, where TGV cells of the 45th and 49th *in vitro* passages were inoculated to newborns at the rate of 10^6 cells/animal, nodules of up to 5 mm in diameter were present at the site of injection 7 days after inoculation in 7/9 mice (46th passage) and 6/6 mice (49th passage). However, these nodules gradually regressed. Mice with regressed tumours were observed for signs of leukaemia after disappearance of the nodule. From the observed group of 11 mice, all eventually died of leukaemia. The mean latency period (98 days) seemed rather longer than in mice injected with cell culture supernatant, though the mean survival time after

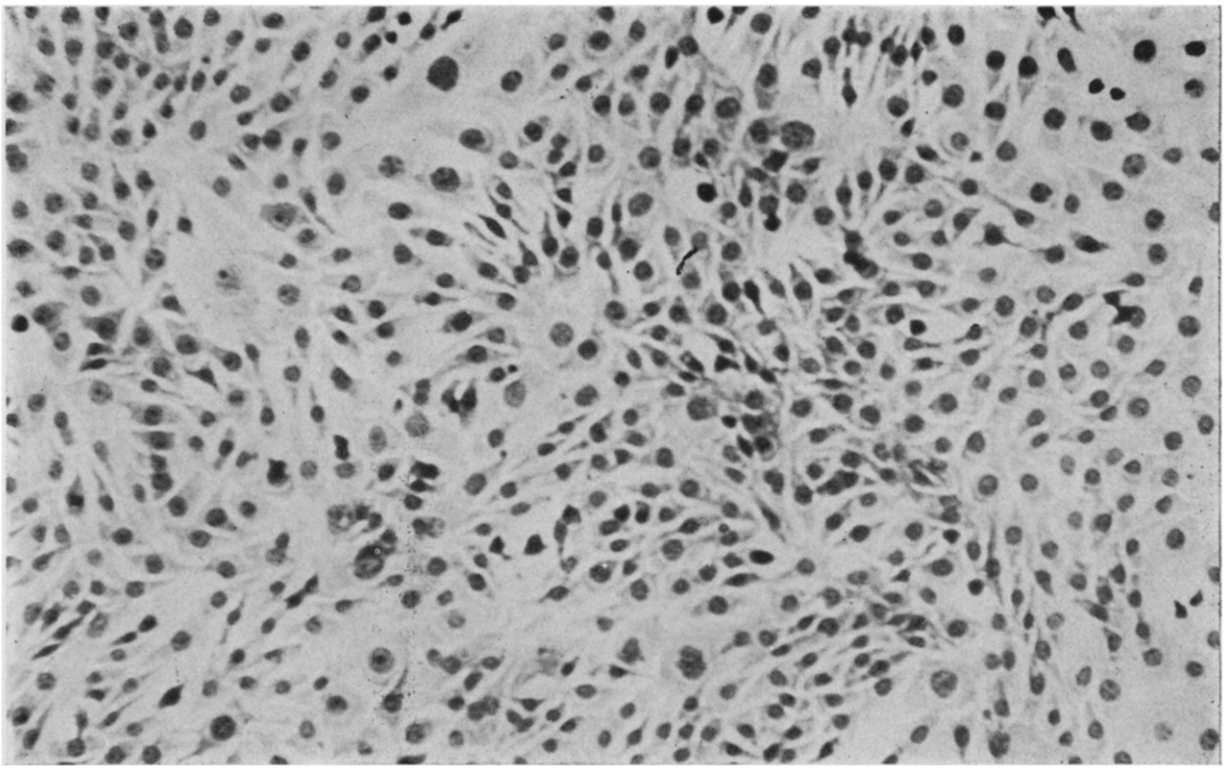


Fig. 1. *TGV cells at the* 34*th* in vitro *passage. Giemsa-stained preparation. Magnification* × 176.

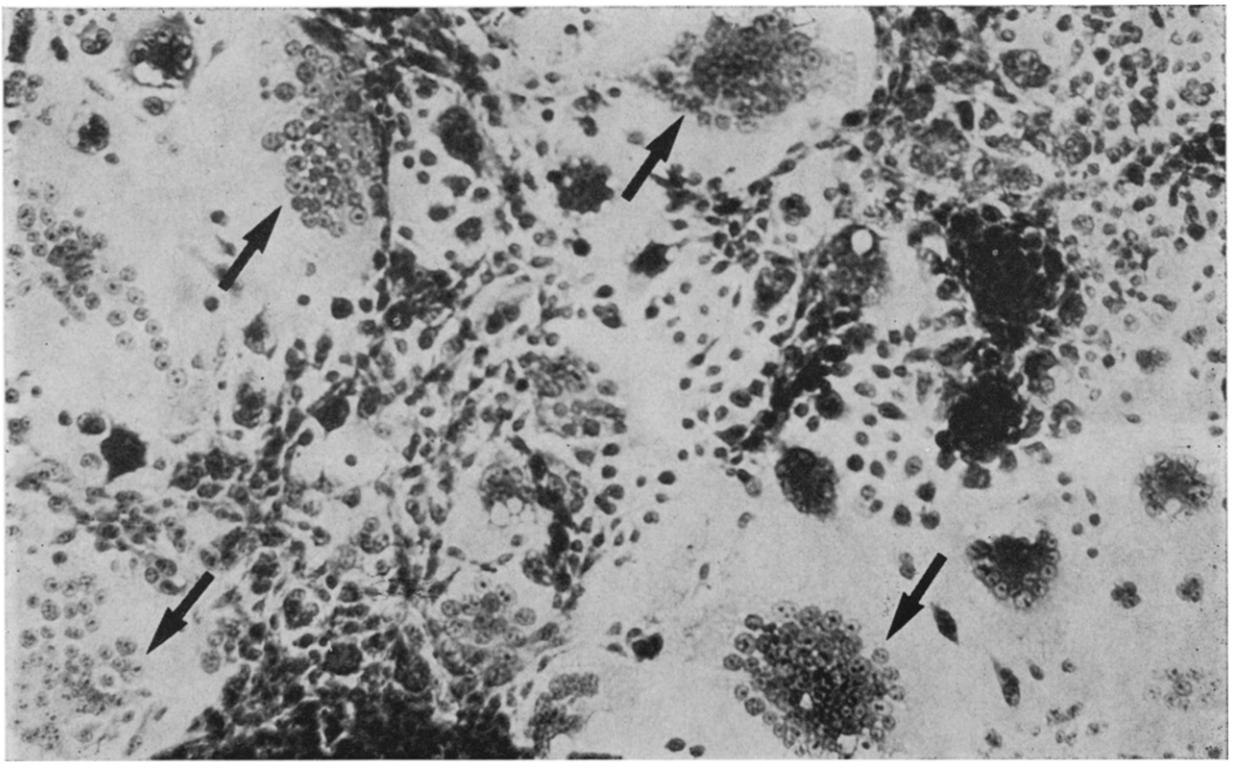


Fig. 2. *TGV cells associated with nearly equal number of XC cells. Aspect of fixed and Giemsa-stained preparation,* 3 *days after association. Foci of giant, degenerating polykaryocytes can be seen* (*arrows*). *Magnification* × 176.

(facing p. 470)

appearance of leukaemia (23 days) was very similar.

DISCUSSION

An *in vitro* adapted cell line (TGV) was developed from explants of a hypertrophied thymus taken from a C3HeB/Fe mouse inoculated as a newborn with the Gross passage A virus and showing typical lesions of lymphatic leukaemia. The TGV line reached a fairly high and constant level of replication (usually 24 hr doubling time) only after several months of stagnation during which little cell proliferation was observed. The initially mixed epithelioid and fibroblast-like cytomorphology of the TGV cell cultures became less conspicuous with time and the cell population reached a rather uniform aspect. Similarly, the differences in cytomorphology between the two derivative clonal sublines K8E and K10F gradually disappeared. In general, both the TGV and its derivatives were quite different in appearance from the mouse lymphoid cell cultures described by other authors [7] as easily grown in suspension. In contrast, the TGV and the derived cells were more or less firmly attached to the glass or plastic surface of the culture vessels throughout the observation period of nearly two years.

In spite of apparently the non-lymphoid character of their growth and morphology, the TGV and derived cell lines constantly reproduced, during at least 22 months, the highly pathogenic Gross leukaemia virus initially present in the explanted thymus. This was supported by convergent data indicating: 1, the constant presence at EM examination of numerous C-type particles, many of them budding on the cell surface or found in the intercellular spaces; 2, high index of immunofluorescence specifically obtained with anti-Gross leukaemia serum; 3, positive XC tests indicating the presence of infectious murine leukaemia virus produced by the TGV cells; and finally, 4, the pathogenic potential of the virus produced, which caused typical lymphoid leukaemia in C3HeB/Fe mice inoculated as newborns with standard samples of culture supernatants. This potential appeared as fairly constant in aliquots harvested at different times between the 2nd and the 51st passage of the cultures (corresponding to the period of 8 to 22 months *in vitro*). This conclusion was reinforced by the observation that the average latency periods before the appearance of the first symptoms of leukaemia and the average survival times of the inoculated mice were quite similar with the different aliquots.

It is significant in this connection, and most probably related to the virus carrier state of the TGV cells, that when these cells were inoculated into syngeneic mice, they grew initially as tumor nodules but regressed rapidly. This leads one to suppose that the immune reaction of the inoculated animals against Gross virus-linked or virus-induced antigens was strong and timely enough to reject the inoculated cells but inefficient in stopping the leukaemogenic process once started. This is reminiscent of data of Doré *et al.* [15] who reported immunization of young adult AkR mice (in spite of their supposed tolerant state toward GLV antigens) with allogenic or syngeneic Gross leukaemia cells.

The exact origin of the cells grown out in our cultures from the thymus explants is not clear and it can be assumed that these cells resulted from a complex process of evolution and selection from a mixed population of connective tissue, epithelial, endothelial and lymphoid cells originally present in the thymus explants. However, it may be admitted that the "stabilized" TGV cultures contained, after one year or more, no typical lymphoid elements and it is significant that the Gross leukemia virus could propagate in these cells without losing a considerable degree of its pathogenic potential.

Imagawa *et al.* [7] also cultivated the pathogenic Gross leukaemia virus but did so in mouse cell cultures of another cytological type which did not adhere firmly to the glass surface and could be passed without trypsinization, a property which these authors felt is common to all leukaemic cells cultivated *in vitro*.

It is, however, worthwhile to emphasize that all the karyological analyses performed on the TGV cell line and its derivatives showed a clear modal number of 40 chromosomes corresponding to the euploid value. This is quite different from the currently observed evolution of *in vitro* adapted mouse cell lines of connective tissue origin characterized by aneuploid karyotypes. Evidently the TGV cells were closer from this point of view to the lymphoid cell lines maintaining fairly well their euploid or nearly euploid chromosomal features [16].

Thus, the relationship between the long-term propagation *in vitro* of the leukaemogenic Gross virus, with maintenance of its pathogenic potential, and the nature of the cells harbouring this virus and sustaining its replication still remains open.

Another problem which has to be clarified, and is currently being studied, is the use of

procedures aiming at the possible production and/or selection *in vitro* in genetically defined host-cell systems, of Gross leukaemia virus variants having a decreased or abolished leukaemogenic potential like the ones obtained previously with the Rauscher leukaemia virus [10, 17].

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REFERENCES

1. A. E. Moore, Growth and persistence of Friend leukemia virus in tissue culture. *J. nat. Cancer Inst.* **30,** 885 (1963).
2. H. Ginsburg and L. Sachs, Long-term cultivation in tissue culture of leukemic cells from mouse leukemia induced by Moloney virus or by X-rays. *J. nat. Cancer Inst.* **27,** 1153 (1961).
3. B. S. Wright and J. C. Lasfargues, Multiplication of the Rauscher leukemia virus in a mixed culture of mouse spleen and thymus cells. *Proc. Amer. Ass. Cancer Res.* **5,** 70 (1964).
4. D. Barbieri, G. Barski and J. K. Youn, Variantes de virus de Rauscher en cultures de cellules d'origine pulmonaire de la souris C57Bl. *C.r. Acad. Sci., Paris,* **256,** 2145 (1967).
5. H. Yoshikura, Y. Hirokawa, Y. Ikawa and H. Subano, Infectious but non leukemogenic Friend leukemia virus obtained after prolonged cultivation *in vitro*. *Int. J. Cancer* **4,** 636 (1969).
6. H. L. Ioachim, L. Berwick and J. Furth, Replication of Gross leukemia virus in long-term cultures of rat thymomas: bioassays and electron microscopy. *Cancer Res.* **26,** 803 (1966).
7. D. T. Imagawa, H. Issa and M. Nakai, Cultivation of Gross virus induced murine thymic lymphoma cells *in vitro*. *Cancer Res.* **28,** 2017 (1968).
8. W. T. Hall, W. F. Andresen and V. J. Evans, *In vitro* and *in vivo* observation on a murine C-type virus. *J. Virol.* **1,** 1238 (1967).
9. G. Barski and J. K. Youn, Protection of mice against Gross leukemia by interfering action of non-leukemogenic C-type murine viruses inoculated into newborns. *J. nat. Cancer Inst.* **47,** 575 (1971).
10. G. Barski, D. Barbieri, J. K. Youn and G. Hue, Low-leukemogenic variants of Rauscher leukemia virus obtained in long-term cultures at supra-optimal temperature. *Int. J. Cancer* **12,** 55 (1973).
11. W. P. Rowe, J. W. Hartley, M. R. Lander *et al.*, Non-infectious AkR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus. *Virology* **46,** 866 (1971).
12. G. Barski, M. G. Blanchard, J. K. Youn and B. Leon, Expression of malignancy in interspecies, Chinese hamster × mouse cell hybrids. *J. nat. Cancer Inst.* **51,** 781 (1973).
13. G. Möller, Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. *J. exp. Med.* **114,** 415 (1961).
14. J. K. Youn, G. Barski and D. Barbieri, Mécanisme d'immunisation préventive à l'aide de cultures de virus de Rauscher à pouvoir pathogène diminué. *Int. J. Cancer* **3,** 116 (1968).
15. J. F. Dore, E. Ajuria et G. Mathe, Immunisation de souris AkR par des cellules de leucémies induites par le virus de Gross: absence de tolérance. *C.r. Acad. Sci., Paris,* **269,** 2479 (1969).
16. W. M. de Bruyn and E. Hansen-Melander, Chromosome studies in the MB mouse lymphosarcoma. *J. nat. Cancer Inst.* **28,** 1333 (1962).
17. G. Barski, J. K. Youn and D. Barbieri, Virus de Rauscher de culture non leucémigène et vaccinant. *C.r. Acad. Sci., Paris,* **264,** 522 (1967).

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The Growth of Human Tumours in Immune Deprived Mice

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Abstract—*The growth of human tumours was studied in immune deprived mice. Immune deprivation was obtained by a combination of thymectomy, whole body irradiation and bone marrow replacement. Five hundred and twenty mice were implanted with 18 different types of tumour from 83 patients. Tumour from 22 of these 83 patients grew in the immune deprived mice. The tumours that grew to a volume that threatened the survival of the mice were carcinomas of the colon, rectum, ovary, kidney and bronchus, and a glioma.*

INTRODUCTION

THE GROWTH and metastasis of human tumours in experimental animals can now be studied by a number of different methods. The recent and more successful methods require suppression of the immunological graft rejection processes. Previous techniques depended upon the fact that in certain areas of the body, such as the cheek pouch in the hamster, or the anterior chamber of the eye in rabbits, a graft was offered partial protection from the immunological rejection processes. In the case of the hamster cheek pouch grafts it was possible to enhance this partial protection by the destruction of lymphocytes using cortisone and antimitotic drugs [1]. A drawback to this latter technique was the necessity to treat with immunosuppressive drugs throughout the experiment. Such drugs could interfere with the growth of the tumour.

Recently, it has been shown that human tumour can become established, and in some cases metastasise spontaneously, in animals in which the graft rejection process has been suppressed, without the need for giving continuous immunosuppressive treatment [2, 3, 4]. Two techniques have emerged and it is not yet clear which is most suitable for human tumour growth. One technique is to use mice with congenital thymic aplasia. These animals are also hairless and are referred to as "nude" mice. The thymic aplasia is the product of a recessive gene and therefore a carefully controlled breeding programme is required. The "nude" mice have so far been used particularly for the *in vivo* culture of carcinoma of the large bowel [2]. The second technique, which can be applied to most rodents, requires thymectomy followed by the destruction of lymphocytes by irradiation antithymocyte serum and cytotoxic drugs.

Tumour implanted into immune deprived animals does not always grow. It is becoming apparent that certain types of tumour are more likely to "take" than others. It is the purpose of this paper to illustrate the types of tumour which are likely to grow in one form of immune deprived animal; that is the mouse prepared by thymectomy, whole body irradiation and bone marrow replacement. Having indicated the tumours that appear to grow most successfully in this preparation the reasons for failure of other classes of tumour are considered

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The possible clinical use of the growth of specimens of patient's tumour in immune deprived animals is discussed.

MATERIALS AND METHODS

Tumour specimens

Tumour specimens reached the laboratory between 10 min and 4 hr after removal from the patient. Tumour areas that appeared to be viable were dissected out. One mm thick slices were cut for transplantation. Adjacent viable tumour was fixed in Bouin's solution and prepared by conventional methods for histological examination. The 1 mm thick slices of the tumour were pared to an area of approximately 9 mm^2 before being transplanted subcutaneously into the right flank of from 4 to 10 mice, depending on the size of the specimen from the patient. The mice were anaesthetized with ether. A total of 520 implantations were made from tumour removed from 83 patients.

Experimental animals

Male and female CBA/lac mice were used. The breeding stock were obtained from the Laboratory Animals Centre, Carshalton, Surrey, England. The mice were given sterile food and water *ad libitum*. Whenever it was thought possible that the growth of the transplant could be effected by sex hormones, the sex of the mouse was chosen to match the sex of the patient.

Technique of immune deprivation

The technique used was based on work published by Miller in 1963 [5]. The mice were thymectomized at 4 weeks of age, 4 weeks later they were given 900 rad whole body irradiation. Irradiation was given at 60 rad per min using a 220 kV X-ray machine, hvl 0·4 mm Cu, focal distance 100 cm. Without a bone marrow graft this dose of irradiation would have been lethal. Within 6 hr of irradiation each mouse received 5×10^6 syngeneic femoral bone marrow cells intravenously. The tumour was implanted between 2 and 4 weeks after the irradiation and bone marrow replacement.

Observation of tumour implanted immune deprived mice

The mice were observed 3–5 times weekly for 9 months, at the end of which time they were killed. Animals were killed before this time if they were ailing or if the tumour had reached a size of 9 mm^3. An extensive necropsy examination was carried out on all mice. A particular search was made for evidence of metastatic involvement of lymph nodes draining the site of implantation and of spread to distant tissues. Tissues with signs of pathological changes were fixed in Bouin's solution. Sections were cut, and stained with haematoxylin and eosin.

Recording tumour growth

The subcutaneously implanted tumours were measured once weekly. Particular note was taken of the tumours at 2 sizes, 75 mm^3 and 9 cm^3. The 75 mm^3 stage was selected because experience showed that this was the smallest tumour volume at which it was possible to be confident that growing tumour was present, rather than a foreign body reaction. The second stage, 9 cm^3, was chosen because this volume approached the volume that would cause debility in the animal.

RESULTS

Subcutaneous tumour implants gaining a volume of 75 mm^3

Twenty-two tumours were established (gained a volume of 75 mm^3) of the 83 tumours implanted (Tables 1 and 2). Unfortunately, with a tumour at the volume of 75 mm^3, it was not possible to obtain a confirmatory biopsy and still allow normal growth of the tumour. However, in 8 mice a tumour of 75 mm^3 was present when the mice were killed at 9 months. In all these animals there was viable tumour. Of the 87 individual implants that gained 75 mm^3 26 subsequently regressed completely before 9 months.

Subcutaneous tumour implants growing to a volume of 9 cm^3

Only 10 of the 22 tumours that grew to a size of 75 mm^3 continued to grow to a volume of 9 cm^3 within the 9 month period (Table 1). These 10 consisted of 3 cases of carcinoma of colon, 3 cases of carcinoma of rectum, 1 case of carcinoma of ovary, 1 case of glioma, 1 case of hypernephroma and 1 case of carcinoma of bronchus. The histological appearance of the tumour removed from the animals was strikingly similar to areas of the respective patients tumours (Figs. 1–6).

Post-mortem findings

In none of the mice examined macroscopically 9 months after implantation, or before 9 months if the tumour had reached 9 cm^3, was there any evidence of metastatic spread of the tumour. Twenty-nine of the 520 implanted mice died before the completion of the experiment from causes apparently unrelated to the

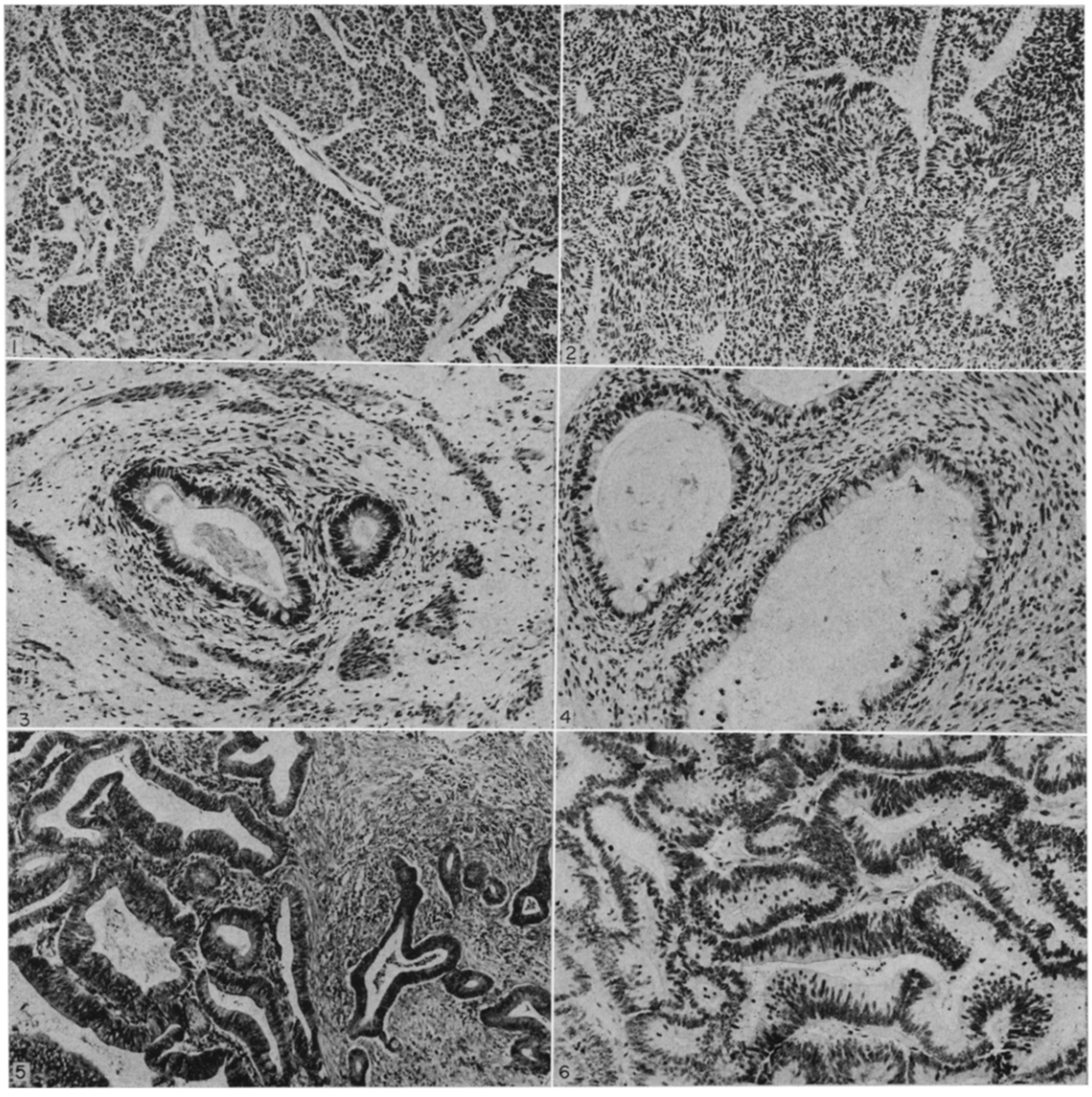


Fig. 1. *Carcinoma of bronchus on removal from the patient* P246 × 150.

Fig. 2. *Carcinoma of the bronchus growing subcutaneously in mice* 6 *months after implantation from the patient whose tumour is illustrated by Fig.* 1. × 150.

Fig. 3. *Teratoma of testes on removal from the patient* P242. × 150.

Fig. 4. *Teratoma growing subcutaneously in mice* 9 *months after implantation from the patient whose tumour is illustrated by Fig.* 3. × 150.

Fig. 5. *Carcinoma of colon on removal from patient* P202. × 150.

Fig. 6. *Carcinoma of colon growing subcutaneously in mice* 5 *months after implantation from the patient whose tumour is illustrated by Fig.* 5. × 150.

(to face p. 474)

Table 1. *Twenty-two tumours growing successfully: Fraction of tumour implants from each patient growing to* 75 mm^3 *and* 9 cm^3; *and the time, post implantation, at which these volumes were reached*

Identity	Tumour	Tumour volume of 75 mm^3	Time taken (months)	Tumour volume of 9 cm^3	Time taken (months)
P 91	Ca. colon	6/10	1–5	5/10	3–7
P 202	Ca. colon	6/6	2–3	6/6	3½–9
P 234	Ca. colon	4/6 (1/6)*	2–4½	3/6	6–8
P 300	Ca. colon	1/5 (1/5)	6	0/5	—
P 224	Ca. rectum	4/6	1–2½	4/6	4–5
P 137	Ca. rectum	6/8 (2/8)	1–1½	3/8	3–6½
P 293	Ca. rectum	6/6	1–1½	4/6	4–5
P 39	Hypernephroma	2/5	2–2½	1/5	8
P 210	Ca. ovary (pseudomucinous)	5/8 (2/8)	3–3½	2/8	7–9
P 88	Ca. ovary (pseudomucinous)	2/6	4–6	0/6	—
P 105	Ca. ovary (granulosacell)	10/10 (6/10)	5–9	0/6	—
P 246	Ca. bronchus	5/5 (2/5)	2–4½	3/5	6–8½
P 303	Ca. bronchus	4/6 (3/6)	3–9	0/6	—
P 338	Glioma	2/5	2–3	2/5	5–6
P 95	Ca. larynx	2/6	3–5	0/6	—
P 337	Ca cervix	3/7 (2/7)	7–9	0/7	—
P 208	Melanoma	5/5 (2/5)	3–9	0/5	—
P 122	Melanoma	1/5	2	0/5	—
P 55	Neuroblastoma	3/5 (1/5)	5–6½	0/5	—
P 146	Teratoma	2/5	2–4	0/5	—
P 242	Teratoma	6/6 (4/6)	2–9	0/6	—
P 181	Fibrosarcoma	2/7	1–5	0/7	—

*The fraction in brackets indicates implants regressing spontaneously before termination of the experiment at 9 months.

Table 2. *Implantation of human tumour in immune deprived mice. Number of patients from whom tumour implants reached a volume of* 75 mm^3 *and* 9 cm^3

Tumour	75 mm^3	9 cm^3
Growth		
Ca. colon	4/5*	3/5*
Ca. rectum	3/5	3/5
Hypernephroma	1/3	1/3
Ca. ovary	3/6	1/6
Ca. bronchus	2/4	1/4
Glioma	1/3	1/3
Ca. larynx	1/5	0/5
Ca. cervix	1/4	0/4
Melanoma	2/7	0/7
Neuroblastoma	1/1	0/1
Teratoma	2/4	0/4
Fibrosarcoma	1/4	0/2
No growth		
Ca. pancreas	0/1	0/1
Ca. breast	0/10	0/10
Ca. bladder	0/3	0/10
Ca. stomach	0/3	0/3
Lymphsarcoma	0/8	0/4
Hodgkin's disease	0/7	0/5

*This fraction is the number of patients from whom successful tumour growth was obtained divided by the total number of patients bearing that particular tumour.

implanted tumour, and most probably related to the animals having received irradiation at a high dose level.

DISCUSSION

Carcinoma of the large bowel will grow with relative ease subcutaneously in immune deprived mice [6]. The implanted tumour frequently grows progressively until it threatens to overwhelm the host. On other occasions the tumour grows for some months and then regresses. In this series of tumours from 83 patients the only other types of tumour that grew as successfully were carcinomas of the ovary, kidney and bronchus, and a glioma. However, this was by no means an exhaustive survey either in numbers and types of tumours or period of post-implantation observation. There is as yet no clear indication why some tumours grow well in immune deprived mice and others not at all. At one time it was thought that the mucin secreted by the ovarian and large bowel tumour cells contributed to their successful growth in the foreign host. Although this may be so, in the later stages of this study non mucin secreting tumours of the large bowel

were observed to proliferate as successfully as those secreting large amounts of mucin. In work to be published elsewhere we have observed with some tumours an extensive polymorph and macrophage infiltration and destruction of the implant within 48 hr of implantation. The reaction was frequently so severe that very few viable tumour cells could be identified. Other tumour implants were found to have provoked no cellular reaction by the host at 48 hr and to be obviously viable.

The changes in volume of an established tumour are the outward manifestation of the delicate balance of cell proliferation and cell death. This balance is tipped towards cell proliferation in the "natural" host of a tumour except in the event of spontaneous regression. It is perhaps not surprising to find that when a tumour is transplanted to a foreign host the delicate balance can become tipped towards cell death. The regressions observed some months after tumours appeared to be established may have been due to a slowly developing immunological rejection [7], or the eventual restraint of tumour growth by the tissue control factors of the host.

Up until the last few years most of our experience with transplanted tumours has been with the rapidly proliferating anaplastic animal tumours. These tumours provide the experimentalist with quick results, but in some cases their relevance to the slowly growing malignant tumours of man must be questioned. If more work is to be done using the early passages of human tumour in immune deprived rodents, we shall have to be prepared to wait months rather than days for results because the implanted human tumour generally grows at much the same rate as is observed in the patient. It will be some years before we are able to relate the behaviour and response to therapy of a patient's tumour growing in a mouse to the likely behaviour of that piece of tumour had it remained in the patient.

REFERENCES

1. H. W. Toolan, Growth of human tumours in cortisone-treated laboratory animals: the possibility of obtaining permanently transplantable human tumours. *Cancer Res.* **13,** 389 (1953).
2. C. O. Povlsen and J. Rygaard, Heterotransplantation of human adenocarcinomas of the colon and rectum to the mouse mutant nude. A study of nine consecutive transplantations. *Acta path. microbiol. scand. Sect. A,* **79,** 159 (1971).
3. L. M. Cobb, Metastic spread of human tumour inplanted into thymectomized, antithymocyte serum treated hamsters. *Brit. J. Cancer* **26,** 183 (1972).
4. L. M. Cobb, E. A. M. Boesen and A. M. Neville, Clear cell carcinoma of the human ovary transplanted to the peritoneal cavity of the hamster. *Transplantation* **16,** 76 (1973).
5. J. F. A. P. Miller, Role of the thymus in recovery of the immune mechanism in the irradiated adult mouse. *Proc. Soc. exp. Biol.* **112,** 785 (1963).
6. L. M. Cobb, The behaviour of carcinoma of the large bowel in man following transplantation into immune-deprived mice. *Brit. J. Cancer* **28,** 400 (1973).
7. P. C. L. Beverley and E. Simpson, Humoral responses to tumour xenografts in ALS-treated mice. *Int. J. Cancer* **6,** 415 (1970).

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Studies on the Sulphate Esters of Dehydroepiandrosterone and Androsterone in the Blood of Women with Breast Cancer

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Abstract—*The concentration of plasma dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) has been measured in* 211 *normal women,* 202 *women with early breast cancer, before and after mastectomy, and in* 90 *patients with advanced breast cancer.*

There was no significant difference between the mean levels of DS or AS in normal women and women with early breast cancer before mastectomy but DS and AS concentrations were significantly subnormal after mastectomy, or in patients with advanced breast cancer.

Plasma DS and AS concentrations were significantly correlated with the urinary excretion of 11*-deoxy-*17*-ketosteroids in all the categories of cancer patients studied.*

INTRODUCTION

STUDIES on plasma 11-deoxy-17-ketosteroids (11DKS) in women with breast cancer have revealed a confused picture, especially in the early disease, with results indicating concentrations lower than [1], equal to [2, 3] or higher [4] than normal. Similar uncertainty seems to exist with regard to urinary 11-DKS excretion [5–11].

These discrepancies may be due, in part, to the effects of stress associated with mastectomy since surgery is known to effect the excretion of urinary steroids [10, 12–15]. The purpose of this study was, therefore, to examine the concentration of plasma dehydroepiandrosterone sulphate (DS) and androsterone sulphate (AS) in women with early breast cancer and to determine the effects of surgery on the plasma levels of these steroids. The opportunity was also taken to compare the plasma DS and AS concentrations in women with advanced breast cancer with those in normal women.

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METHODS

Subjects

The control group consisted of 211 ostensibly normal women who volunteered to give blood. There were 202 patients with early breast cancer (Stage I and II), the majority of whom had blood taken the day before operation and 10–14 days after radical mastectomy or wide excision. There were 90 women with advanced breast cancer. Diagnosis was confirmed histologically in all cases. In analysing the results the various groups of women have been divided into age decades and the mean age and standard deviation for control women and breast cancer patients in each of the decades are similar.

Plasma was obtained from blood taken between 10 a.m. and 8 p.m. into heparinized tubes and stored at −20° until analysed. Analysis of plasma from 9 subjects showed no significant variation in either plasma DS or AS levels between 10 a.m. and 5 p.m. (The mean change was 6% and 1% respectively). Urine was collected for the 24 hr, during which the blood

was taken, and stored at −20°C. The measurement of 17-hydroxycorticoids and 11-DKS was carried out using the methods previously described [5].

Method for determination of plasma DS and AS

To each 2 ml plasma was added pregnenolone sulphate and dihydrotestosterone sulphate as internal standards. The loss of these compounds through the method are the same as that for DS or AS [17]. The plasma was extracted twice with 4 ml ethanol and the pooled ethanol left overnight, after which it was centrifuged (600 g × 10 min). The decanted ethanol extract was evaporated under N_2 and to the dried extract was added water (0·05 ml) and 1 ml MeOH (containing 0·02 M NaCl) and the mixture was left at 50° for 30 min in a glass-stoppered tube to dissolve the steriod esters. To this solution was added 1 ml $CHCl_3$ and the mixture chromatographed on a 4 g Sephadex LH20 column (i.d. 1 cm) using the solvent MeOH: $CHCl_3$ (1:1 v/v) containing 0·01 M NaCl. The fraction containing the steroid monosulphates was dried and solvolysed and the steroids purified on an alumina column (4 g containing 8% water) using benzene containing 1% ethanol. The fraction containing DHA, androsterone, pregnenolone and dihydrotestosterone was evaporated to dryness and partitioned between 1 ml water containing 5% ethanol and 5 ml isopentane. After evaporation of the isopentane layer, the steroids were estimated by GLC as previously described [17]. Results are expressed as μg of the hydrolysed steroid per 100 ml plasma.

For each set of six plasmas estimated, one quality control plasma was included. Estimations were repeated when the results from the quality control differed by more than 15% from the accepted mean value or where the results using the internal standards dihydrotestosterone and pregnenolone differed by more than 15%.

RESULTS

Early disease

(*a*) *Effect of mastectomy on plasma DS and AS levels.* Mastectomy is associated with a marked

Table 1. *Logarithmic mean levels of plasma DS and AS of normal women and patients with early breast cancer before and after surgery*

Age		33–40			41–50			51–60			61–70			⩾71		
		Mean*	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*
Normal	DS	2·029	0·188	54	1·917	0·278	55	1·803	0·224	40	1·688	0·259	43	1·527	0·329	19
	AS	1·521	0·227	54	1·442	0·390	55	1·381	0·293	40	1·304	0·291	43	1·199	0·354	19
Pre-operative	DS	2·005	0·202	19	1·846	0·260	63	1·773	0·274	48	1·646	0·305	52	1·535	0·317	20
	AS	1·490	0·305	19	1·476	0·309	63	1·379	0·302	48	1·258	0·365	52	1·144	0·265	20
Post-operative	DS	1·888	0·225	19	1·721	0·255	62	1·574	0·288	44	1·488	0·284	48	1·486	0·268	16
	AS	1·174	0·377	19	1·168	0·324	62	1·014	0·278	44	1·036	0·384	48	0·991	0·316	16

N = the number of subjects.

*The concentrations are expressed as $\log_{10}$ μg/100 ml plasma.

Table 2. *Statistical comparison of the logarithmic mean levels of plasma DS and AS of control women and patients with early breast cancer before and after surgery*

Age		33–40			41–50			51–60			61–70			⩾71		
		t	*P*	*N*	*t*	*P*	*N*	*t*	*P*	*N*	*t*	*P*	*N*	*t*	*P*	*N*
Control vs Pre-operative	DS	0·46	N.S.	73	1·42	N.S.	118	0·54	N.S.	88	0·73	N.S.	97	0·73	N.S.	39
	AS	0·48	N.S.	73	0·52	N.S.	118	0·02	N.S.	88	0·68	N.S.	97	0·56	N.S.	39
Control vs Post-operative	DS	2·68	<0·01	73	4·16	<0·001	117	4·04	<0.001	84	3·51	<0.001	91	0·40	N.S.	35
	AS	4·78	<0·001	73	4·16	<0·001	117	5·89	<0·001	84	3·72	<0·001	91	1·83	N.S.	35
Pre-operative vs Post-operative	DS	2·81	<0·02	38	5·99	<0·001	125	3·44	<0·01	92	4·54	<0·001	100	2·18	<0·05	36
	AS	4·81	<0·001	38	7·65	<0·001	125	7·19	<0·001	92	5·22	<0·001	100	2·05	N.S.	36

N = the sum of the number of subjects.

decrease in the plasma level of both DS and AS (Table 1). The differences between these levels before and after mastectomy are significant in all age groups except for plasma AS concentrations in women aged more than 71 years (Table 2). Plasma AS level was proportionally more depressed after mastectomy than DS (paired t-test $t=5{\cdot}69$; $P<0{\cdot}001$; $N=191$).

Androgen levels of plasma taken 5–15 days after mastectomy indicated that the decrease in AS and DS concentrations was similar within these times.

(*b*) *Comparison of plasma DS and AS concentration with that in normal women.* The mean post-operative plasma DS and AS levels were significantly lower than those of normal controls (except in women aged 71 and over), but the pre-operative concentrations, while lower than those of controls, were not significantly subnormal (Tables 1 and 2, and Fig. 1).

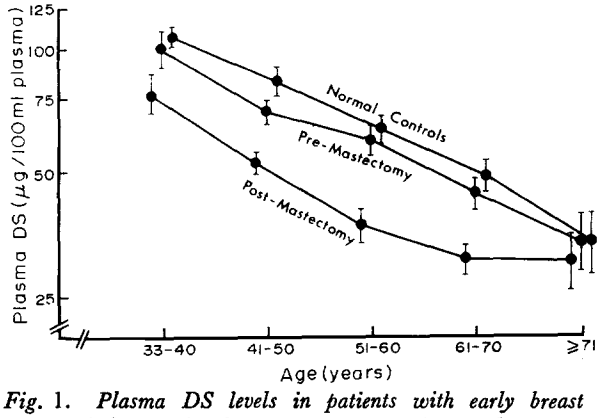


Fig. 1. *Plasma DS levels in patients with early breast cancer, before and after mastectomy, and normal women.*

The mean plasma levels are shown for DS and the S.E.M. for the various age groups studied. Stastical data are given in Table 2.

Advanced disease

The mean plasma level of DS and AS in patients with advanced breast cancer was significantly lower than that in all control age groups except for DS in women aged more than 61 years (Fig. 2 and Table 3).

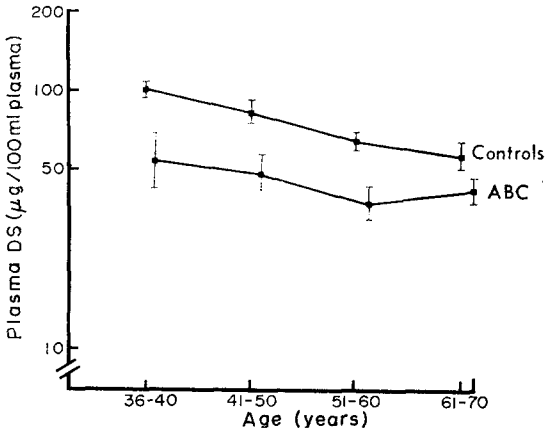


Fig. 2. *Plasma DS levels in patients with advanced breast cancer and normal women.*

The mean plasma levels are shown for DS and the S.E.M. for the various age groups studied. Statistical data are given in Table 3.

Relationship between plasma and urinary ketosteroids

In all categories of patients studied and in the controls, the plasma DS and AS concentrations are highly correlated with 11D-KS excretion and with the ratio of 11-DKS and 17-hydroxycorticoids (used as a prognostic measure for patients treated by endocrine ablation [18]. Also the slopes and intercepts of the regression lines for DS (or AS) against 11-DKS excretion were statistically indistinguishable (Tables 4 and 5, and Fig. 3).

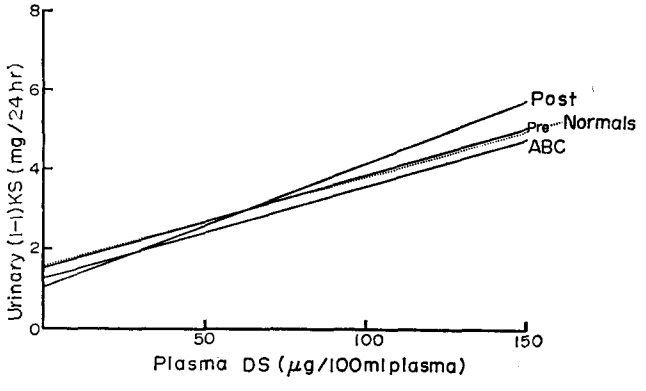


Fig. 3. *Relationship between plasma DS concentration and urinary excretion of* 11-*DKS*

The regression lines are shown for urinary 11-*DKS excretion on plasma concentration of DS for patients with early breast cancer (before (PRE) and after (POST) mastectomy), advanced breast cancer (ABC) and normal women. The statistical data are given in Table* 4.

Table 3. The logarithmic mean levels of plasma DS and AS and their statistical comparison of normal women and subjects with advanced breast cancer

(*a*) Mean DS levels

Age	36–40				41–50				51–60				61–70			
	Mean*	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*
Control	1·998	0·171	33	3·22	1·917	0·278	55	3·13	1·803	0·224	40	3·65	1·746	0·256	28	1·97
Cancer	1·729	0·402	13	<0·01	1·697	0·382	33	<0·01	1·559	0·322	26	<0·001	1·602	0·217	18	N.S.

(*b*) Mean AS levels

Age	36–40				41–50				51–60				61–70			
	Mean*	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*	Mean	S.D.	*N*	*t*/*P*
Control	1·489	0·215	33	2·16	1·442	0·390	55	2·306	1·381	0·293	40	2·89	1·311	0·277	28	2·72
Cancer	1·269	0·484	13	<0·05	1·226	0·485	33	<0·05	1·126	0·426	26	<0·01	1·050	0·371	18	<0·01

*The concentrations are expressed as $\log_{10}$ μg/100 ml plasma.

Table 4. Linear regression equations of urinary 11-deoxy-17-ketosteroids (y) *and blood DS or AS* (x)

Clinical status	*x*	Slope	Inter-cept	*N*	*r*
Controls	DS	0·0231	1·482	55	0·647*
	AS	0·0293	2·459	55	0·381†
Early breast cancer before surgery	DS	0·0240	1·446	189	0·594*
	AS	0·0401	1·624	212	0·466*
Early breast cancer after surgery	DS	0·0314	1·029	193	0·655*
	AS	0·0666	1·335	212	0·524*
Advanced breast cancer	DS	0·0234	1·256	93	0·736*
	AS	0·0438	1·563	84	0·631*

Urinary 11-deoxy-17-ketosteroids are expressed as mgm/24 hr and DS (or AS) as μg/100 ml plasma.
*$P<0·001$
†$P<0·01$

*Table 5. Linear regression equations of urinary steroid ratio** (y) *and blood DS or AS* (x)

Clinical status	*x*	Slope	Inter-cept	*N*	*r*
Early breast cancer before surgery	DS	0·002	0·236	211	0·432†
	AS	0·003	0·309	211	0·287‡
Early breast cancer after surgery	DS	0·002	0·233	211	0·417†
	AS	0·005	0·270	211	0·328†
Advanced breast cancer	DS	0·002	0·186	87	0·575†
	AS	0·004	0·204	86	0·568†

*Ratio of 11-DKS (mg/24 hr) to 17-OHCS (mg/24 hr)
†$P<0·001$
‡$P<0·01$

DISCUSSION

The results show that the concentration of plasma DS or AS in women with early breast cancer 5 – 15 days after mastectomy, or in women with advanced breast cancer are lower than those in normal women. In contrast, the concentration of plasma DS and AS shortly before mastectomy is within the normal range. The latter result is in general agreement with the findings of Brownsey *et al.* [3]. These authors did not state when blood was taken from their patients but from our results it would appear that pre-operative specimens were used.

It would be expected from the close correlation of DS or AS with urinary 11-DKS (Fig. 3) that the latter would also be depressed after surgery. Analysis of 167 patients showed that this was indeed the case ($t=5·27$; $P<0·001$) The effects associated with mastectomy on urinary and plasma 11-DKS do not appear to have been fully appreciated and may account for the diverse results reported in literature [2, 3, 5, 9–11, 19]. Surgery, other than mastectomy, is associated with changes in urinary 11-DKS similar to those found with mastectomy [13].

The reason for the decrease in plasma DS and AS concentrations after breast surgery are not known and the question arises whether the normal basal concentration of steroids are better reflected by the pre-operative or the post-operative levels. Quite clearly, women before operation cannot be regarded as "normal women" and it is more than likely that some

endocrine changes occur on entry into hospital with bed-rest and with justified apprehension. For example, urinary 17 OHCS excretion is significantly higher before mastectomy than afterwards ($t=2{\cdot}45$; $P<0{\cdot}02$; $N=167$) and it has already been shown that the post-operative 17OHCS excretion, is within, or somewhat higher than the normal range. This implies a measurable degree of stress before operation and would mean that pre-operative DS and AS levels would almost certainly be above basal levels. On the other hand there can be no doubt, that surgery and anaesthesia depress urinary excretion of androgen metabolites, and therefore the post-operative DS and AS values are probably also not a true reflection of basal levels.

Recent findings in a prospective study have shown that approximately half of the women who subsequently developed breast cancer excreted subnormal amounts of urinary androgen metabolites before diagnosis [20–22]. This result suggests that the post-mastectomy levels found in patients with the early disease may be a better indicator of pre-diagnostic basal levels than measurements made before mastectomy. Further prospective studies are required for certainty on this point.

The subnormal levels of plasma DS and AS in women with advanced breast cancer reported here are in accord with previously published results on 11-DKS in blood and urine [1, 3, 6, 22–24]. It has been suggested that the subnormal amounts of urinary 11-DKS found in women with advanced breast cancer are due to changes in metabolism associated with illness and not to a lower production rate [25]. Our present results show a decreased level of plasma 11-DKS (not due to changes in plasma protein binding [26]) in women with advanced breast cancer. This is not in accord with the above hypothesis and our results agree with the report of Poortman *et al.* [27] that the urinary production rates of dehydropeiandrosterone and DS were significantly depressed in women with breast cancer who were studied several years after mastectomy and while free of the disease.

It is obvious that the study of the endocrinology of breast cancer is complicated by the large number of variables involved. Age, bed-rest, diet, drug, stress, weight, height, race and menopausal status have all been shown to affect endocrine function [28–30]. This study shows that the time before or after mastectomy is another important factor.

REFERENCES

1. N. Deshpande, J. L. Hayward and R. D. Bulbrook, Plasma 17-hydroxy-corticosteroids and 17-oxosteroids in patients with breast cancer and in normal women. *J. Endocr.* **32,** 167 (1965).
2. D. Y. Wang, Plasma androgens in breast cancer. In *The Human Adrenal Gland in Relation to Breast Cancer* (Edited by K. Griffiths and E. H. D. Cameron) p. 71. Alpha Omega Alpha, Cardiff. (1969).
3. B. Brownsey, E. H. D. Cameron, K. Griffiths, E. N. Gleave, A. P. M. Forrest and H. Campbell, Plasma dehydroepiandrosterone sulphate levels in patients with benign and malignant breast disease. *Europ. J. Cancer,* **8,** 131 (1972).
4. H. Benard, J. S. Bourdin, R. T. Saracino and A. Seeman, Etudes des 17-cetosteroides plasmatiques dans 51 cas de cancer du sein. *Ann. Endocr. Paris* **23,** 525 (1962).
5. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, Abnormal excretion of urinary steroids by women with early breast cancer. *Lancet* **ii,** 1238 (1962).
6. G. Bacigulupo and H. Lingk, Die Urinausscheidungen von Neutralen 17-ketosteroiden, Androsteron und Aetiocholanolon bei Gesunden Frauen und Frauen mit Fruhem und Vorgeschrittenem Brustkrebs. *Arch. Geschwulstforsch* **32,** 95 (1968).
7. R. M. Gutierrez and R. J. Williams, Excretion of ketosteroids and proneness to breast cancer. *Proc. nat. Acad. Sci.* **59,** 938 (1968).
8. J. Marmorston, Urinary hormone metabolite levels in patients with cancer of the breast, prostate and lung. *Ann. Acad. Sci. (N.Y.)* **125,** 959 (1966).
9. E. H. D. Cameron, K. Griffiths, E. N. Gleave, H. J. Stewart, A. P. M. Forrest and H. Campbell, Benign and malignant breast disease in South Wales: a study of urinary steroids. *Brit. med. J.* **4,** 768 (1970).
10. D. Y. Wang, M. C. Swain, J. L. Hayward and R. D. Bulbrook, Hormones in the aetiology and clinical course of breast cancer. *Rec. Res. Cancer Res.* **39,** 177 (1972).

11. A. P. Wade, J. C. Davis, M. C. K. Tweedie, C. A. Clarke and B. Haggart, The discriminant function in early carcinoma of the breast. *Lancet* **i,** 853 (1969).
12. H. Tanaka, H. Manabe, K. Koshiyama, Y. Hamanaka, K. Matsumoto and T. Uozumi, Excretion patterns of 17-ketosteroids and 17-hydroxycorticosteroids in surgical stress. *Acta endocr. (Kbh)* **65,** 1 (1970).
13. J. D. Hardy, E. M. Richardson and F. C. Dohan, The urinary excretion of corticoids and 17-ketosteroids following major operations. *Surg. Gynec. Obstet.* **96,** 448 (1953).
14. G. W. Thorn, D. Jenkins and J. C. Laidlaw, The adrenal response to stress in man. *Rec. Progr. Hormone Res.* **8,** 171 (1953).
15. J. A. Nicholas, P. D. Wilson and C. J. Umberger, Observations on adrenocortical function in patients undergoing operations upon the bones and joints. *Surg. Gynec. Obstet.* **99,** 1 (1954).
16. A. Halme, A. Pekkarinen and M. Turunen, On the excretion of noradrenaline, adrenaline, 17-hydroxycorticosteroids and 17-ketosteroids during the post-operative stage. *Acta endocr. (Kbh) Suppl.* **32.**
17. D. Y. Wang, R. D. Bulbrook, B. S. Thomas and M. Friedman, Determination of solvolysed sulphate esters of dehydroepiandrosterone and androsterone in human peripheral plasma by gas–liquid chromatography. *J. Endocr.* **42,** 567 (1968).
18. H. Miller, J. A. Durant, A. G. Jacobs and J. F. Allison, Alternative discriminating function for determining hormone dependency of breast cancer. *Brit. med. J.* **1,** 147 (1967).
19. R. D. Bulbrook and J. L. Hayward, Endocrine studies in patients with early breast cancer. In *Current Concepts in Breast Cancer* (Edited by A. Segaloff, K. K. Meyer and S. DeBakey) p. 115. Williams & Wilkins, Baltimore (1967).
20. R. D. Bulbrook and J. L. Hayward, Abnormal urinary steroid excretion and subsequent breast cancer. *Lancet* **i,** 519 (1967).
21. R. D. Bulbrook, J. L. Hayward and C. C. Spicer, Relation between urinary androgen and corticoid excretion and subsequent breast cancer. *Lancet* **ii,** 395 (1971).
22. R. D. Bulbrook and J. L. Hayward, Endocrine studies in patients with advanced breast cancer. In *Current Concepts in Breast Cancer* (Edited by A. Segaloff, K. K. Meyer and S. DeBakey) p. 139. Williams & Wilkins, Baltimore (1967).
23. R. D. Bulbrook, J. L. Hayward, C. C. Spicer and B. S. Thomas, A comparison between the urinary steroid excretion of normal women and women with advanced breast cancer. *Lancet* **ii,** 1235 (1962).
24. S. Kumaoka, N. Sakauchi, O. Abe, M. Kusama and O. Takatani, Urinary 17–ketosteroid excretion of women with advanced breast cancer. *J. clin. Endocr.* **28,** 667 (1968).
25. B. Zumoff, H. L. Bradlow, T. F. Gallagher and L. Hellman, Decreased conversion of androgens to normal 17-ketosteroid metabolites: a non-specific consequence of illness. *J. clin. Endocr.* **32,** 824 (1971).
26. D. Y. Wang and R. D. Bulbrook, The binding of steroids to plasma proteins in normal women and women with breast cancer. *Europ. J. Cancer* **5,** 247 (1969).
27. J. Poortman, J. H. H. Thijssen and F. Schwartz, Androgen production and conversion to oestrogens in normal post-menopausal women and in selected breast cancer patients. *J. clin. Endocr.* **37,** 101 (1973).
28. A. Hendrikx, W. Heyns, O. Steeno and P. de Moor, Influence of body size, changes in body weight and/or food intake on urinary excretion of 11-desoxy-17-ketosteroids. In *Proc. 2nd Symp. Steroid Hormones, Ghent,* p. 63. (Edited by A. Vermeulen) Inter. Congress Series No. 101. Excerpta Medica Foundation (1965).
29. R. D. Bulbrook, In *Clinical Evaluation in Breast Cancer* (Edited by J. L. Hayward and R. D. Bulbrook) p. 77. Academic Press, New York (1966).
30. M. B. Lipsett, H. Wilson, M. A. Kirschner, S. G. Korenman, L. M. Fishman, G. A. Sarfaty and C. W. Bardin, Studies on Leydig cell physiology and pathology: secretion and metabolism of testosterone. *Rec. Progr. Horm. Res.* **22,** 245 (1966).

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Squamous Metaplasia and Respiratory Tumors Induced by Intratracheal Installations of 7, 12-Dimethylbenz(a)-Anthracene in Syrian Golden Hamsters

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Abstract—*Repeated intratracheal installations of 7,12-dimethylbenz(a)anthracene in the respiratory tract of Syrian golden hamsters induced papillomas, carcinoma* in situ *and squamous cell carcinomas of the larynx and trachea. The neoplastic progression was characterized by pronounced and extensive squamous metaplasia showing evidence of hyperortho- and hyper-parakeratosis, without secondary changes, inflammation and fibrosis in the lung. The total number of tumors seen,* 20 *and* 13 *respectively, the short latent period* (20 *or* 21 *weeks*) *and the low doses,* 0·85 *mg and* 1·2 *mg in total respectively, show that DMBA is also a potent carcinogen in the respiratory system.*

7, 12-Dimethylbenz (a) anthracene (DMBA) has been used repeatedly in studies on tumor induction in the respiratory system [1–5]. The neoplastic response in studies of the lung was, however, limited, and a number of unspecific changes, chronic pulmonitis and early death, ensued. DMBA among other polycyclic hydrocarbons is found in gasoline exhaust [6] and, therefore, deserves attention as a potential environmental hazard. Intratracheal instillations of polycyclic hydrocarbons in Syrian golden hamsters have been shown to be effective tumor inducers without being associated with significant harmful side effects [7]. To evaluate the carcinogenic potential of DMBA for the respiratory system as well as the neoplastic development, we have studied the response of the Syrian golden hamster to intratracheal instillations of DMBA.

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MATERIALS

DMBA and hematite (ferric oxide, Fe_2O_3) were obtained from Fisher Scientific Company, Fair Lawn, New Jersey and saline solution (0·9% sodium chloride, sterile nonpyrogenic) from Baxter Laboratories, Inc., Morton Grove, Illinois.

Animals

One-hundred twenty-six male Syrian golden hamsters from the randomly bred colony of this Institute were used. They were kept in plastic cages, fed Wayne diet pellets (Allied Mills, Chicago, Illinois) and water *ad libitum.*

Treatment

One hundred μg DMBA and 100 μg Fe_2O_3 (group 1) and 50 μg DMBA and 50 μg Fe_2O_3 (group 2) as mixed dusts were administered intratracheally once a week for an average of 12–17 weeks in saline suspensions. Before treatment the animals were anesthesized with

a 0·4 ml of 1% solution of Brevital Sodium (sodium-α-DL-1-methyl-5-allyl-5[1-methyl-2-pentynyl] barbiturate) (Eli Lilly and Co., Indianapolis, Ind.) injected intraperitoneally. After treatment all animals were observed for general appearance, frequency of breathing, etc., and weighed weekly. The animals were allowed to die spontaneously or were killed when moribund. Complete autopsies were performed on all animals. All organs showing gross abnormalities as well as all tumors were examined histologically. The lungs were excised fully expanded with the trachea and mediastinal organs included. The whole organ was fixed in 10% buffered formalin. Sections from all lobes and trachea were embedded in paraffin and stained with hematoxylin eosin, as well as other stains when needed.

RESULTS

Within a few weeks after starting treatment the animals showed respiratory problems, increased frequency of breathing and dyspnea. A continously progressing general deterioration of the physical condition also occurred with weight loss, anorexia and a high mortality throughout the experiment (Table 1). The most common cause of death, apart from general deterioration resulting from the toxic effects of DMBA, was respiratory problems and ultimately suffocation caused by the respiratory tumors. Both groups, regardless of dose, showed the same gross as well as histological changes.

Animals autopsied during the initial weeks of treatment already showed extensive lesions in the larynx, trachea and lungs. Among the first visible alterations in the trachea were fragmentation and dissolution of columnar cells, slit formation and expulsion of the superficial mucosa. Basal cell hyperplasia with piling up of enlarged basal cells was a frequent occurrence. A characteristic feature of animals dying at all stages of the experiment was extensive squamous metaplasia. The epithelium consisted of several layers of regularly built squamous cells with a marked tendency to keratin formation. The epithelial cells were eosinophilic with intercellular bridges and keratin droplets in the cytoplasm. The surface was covered with a distinct layer of parakeratotic and dyskeratotic keratin, increased alcian blue and PAS staining and increased content of certain amino groups like $-NH_2$ and -SS groups were seen.

In the larynx, the regular squamous epithelium was thickened and covered by hyperkeratotic and parakeratotic material. In the lungs, the alterations were milder at the beginning with slight metaplasia of the bronchial epithelium and adenomatous hyperplasia of the peripheral epithelium in two animals. Inflammatory changes were mild; a few stromal lymphocytes as well as occasional accumulations of granulocytes in the tracheal stroma were seen in a few animals.

Three months after starting the experiment the first distinct neoplasms occurred in the respiratory system. They were papillomas composed of proliferating squamous epithelium and scanty connective tissue. These tumors

Table 1. *Total number of animals and tumors*

Group	Treatment	Total No. of animals	Animals alive at weeks 10	20	30	40	60	80	100	Effective No. of animals	Total No. TBA	Total No. Resp. TBA	Total No. Resp. tumors	Larynx Papilloma	Larynx Ca *in situ*	Larynx Sq. cell ca.	Trachea and main bronchi Papilloma	Trachea and main bronchi Ca *in situ*	Trachea and main bronchi Sq. cell ca.	Lung Adenoma	Lung Adenoca.
I	100 μg DMBA + 100 μg Fe_2O_3	48	38	18	4	—	—	—	—	46	17	15	20	3	1	2	5	1	5	3	–
II	50 μg DMBA + 50 μg Fe_2O_3	30	26	19	5	—	—	—	—	28	13	11	13	2	1	–	7	–	2	–	1
III	Untreated Control	48	48	47	43	38	20	12	0	48	3	—	—	–	–	–	–	–	–	–	–

TBA = tumor bearing animals.

were most frequent (Table 1), occurring in 17 of 34 animals and were histologically similar in the larynx and trachea. The epithelial proliferation was occasionally extensive with cytological irregularities comparable to a carcinoma *in situ*.

Malignant massive neoplasms were seen both in the trachea and larynx 14 weeks after starting treatment. They were mainly located in the proximal parts of the respiratory system with only one peripheral tumor. The tumors were all of squamous origin. They consisted of proliferating squamous epithelium infiltrating the tracheal wall forming epithelial islands surrounded by a fibrotic stroma. Some of these lesions occasionally attained a large size completely obstructing the lumen.

The neoplastic progression in the lung was not so obvious as in the proximal parts of the respiratory system. Congestion and edema occurred occasionally as well as adenomas in 3 animals. In one animal an adenocarcinoma composed of papillary structures containing monomorphic hyperchromatic cells was seen.

DISCUSSION

These results show that DMBA is one of the most effective agents in inducing tumors of the respiratory system of Syrian golden hamsters when compared to other intratracheally instilled chemicals [7–11]. Experiments with polycyclic hydrocarbons such as benzo(a)pyrene (B(a)P) used much higher doses, 0·5–3 mg per application [10, 11]. In our experiment the dose was much lower, 50 μg per application. The potency of DMBA is further emphasized by the large number of tumors seen, 15 in group 1 and 11 in group 2. The toxic effect was significant as seen from the mortality figures, all animals were dead at 40 weeks. Previous studies using intratracheally instilled DMBA [1] showed even stronger toxic effects compared to the neoplastic response. The toxic effects were also obvious in experiments using intratracheal instillation of DMBA in rats [12].

The progression leading to the formation of tumors in the respiratory system in these experiments was characterized by marked squamous metaplasia. Squamous metaplasia is a common occurrence after intratracheal instillations of polycyclic hydrocarbons. Saffiotti *et al.* [11] showed in their study that 8 of 30 male animals and 6 of 30 female animals had squamous metaplasia alone. Harris *et al* [13] using a B(a)P/Fe_2O_3 mixture reported that squamous metaplasia and hyperplasia occurred only when both agents were used together. Ferric oxide alone produced a marked basal cell hyperplasia which was reversible in all animals.

The tumors seen in this study were also of squamous origin, papillomas as well as squamous cell carcinomas. Squamous cell carcinomas have been closely linked with cigarette smoking based on studies showing an increase in the rate of squamous cell carcinomas among cigarette smokers [13].

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REFERENCES

1. G. Della Porta, L. Kolb and P. Shubik, Induction of tracheobronchial carcinomas in the Syrian golden hamster. *Cancer Res.* **18,** 592 (1958).
2. P. Gross, E. Tolker, M. A. Babyak and M. Kaschak, Experimental lung cancer in hamsters. Repetitive intratracheal applications of two carcinogenic hydrocarbons. *Arch. Environ. Health* **11,** 59 (1965).
3. L. N. Pylev, Experimental induction of lung cancer in rats by intratracheal introduction of 9,10-dimethyl-1,2-benzanthracene. *Bull. exp. Biol. Med.*, **52,** 99 (1961).
4. L. N. Pylev, Induction of lung cancer in rats by intracheal insufflation of carcinogenic hydrocarbons. *Acta Un. int. Cancer* **19,** 688 (1962).
5. L. M. Shabad, Experimental cancer of the lung. *J. nat. Cancer Inst.* **28,** 1305 (1962).
6. G. Grimmer, A. Hildebrandt and H. Bohnke, Probenahme und Analytik polycycyklischer aromatischer Kohlenwasserstoffe in Kraftfahrzeugen. *Brennstoff-Chemie*, **25,** 531 (1972).
7. U. Saffiotti, F. Cefis and L. H. Kolb, A method for the experimental induction of bronchogenic carcinoma. *Cancer Res.* **28,** 104 (1968).
8. U. Saffiotti, Experimental respiratory tract carcinogenesis. In *Prog. exp. Tumor Res.* (Edited by F. Homburger) Vol. 11, pp. 302–333. Karger, Basel. (1969).

9. U. Saffiotti, Experimental respitatory tract carcinogenesis and its relation to inhalation exposures. In *Inhalation Carcinogenesis*, pp. 27–51. AEC Symp. Series 18, Div. Techn. Info., U.S. Atomic Energy Commission. (Edited by M. G. Hanna, Jr., P. Nettesheim and J. R. Gilbert) Oak Ridge, Tennessee (1970).
10. U. Saffiotti, R. Montesano, A. R. Sellakumar and D. G. Kaufman, Hamster respiratory carcinogenesis induced by different dose levels of benzo (a)pyrene and ferric oxide. *J. nat. Cancer Inst.* **49,** 1199 (1972).
11. A. Sellakumar and P. Shubik, Carcinogenicity of 7H-dibenz(cg) carbazole in the respiratory tract of hamsters. *J. nat. Cancer Inst.* **48,** 1641 (1972).
12. W. H. Blair and H. Rao, Development of lung neoplasms in rats treated with 7,12-dimethylbenz(a)anthracene. *Proc. Amer. Assoc. Cancer Res.* Vol. 14, Abstract 497 (1973).
13. L. Kreyberg, The significance of histological typing in the study of the epidemiology of primary epithelial lung tumors: A study of 466 cases. *Brit. J. Cancer.* **8,** 199 (1954).

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Blood Group Substances (BG) as Carcinofetal Antigens in Carcinomas of the Distal Colon

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Abstract—*Colonic adenocarcinomas were studied for the presence of blood group substances (BG) using the mixed cell agglutination reaction (MCAR). Forty five to 68% of colonic carcinomas contained BG-like substances while the surrounding non-neoplastic mucosa was BG-negative in the distal large bowel (descending colon, pelvic colon, rectum). Colonic polyps without histological signs of malignancy did not contain BG-like activity. In addition, BG reactivity was present in metastases from colonic carcinomas to the liver. Since the mucosal cells of the whole colon contain BG in the fetal period which, however, rapidly disappear after birth, reappearance of BG in the tumor may represent derepression of embryonic gene functions during carcinogenesis. Therefore, normal antigens like BG behave as "carcinofetal antigens" in certain carcinomas of the distal large bowel.*

INTRODUCTION

SINCE the first description of an antigenic relationship between human tumors and fetal tissues by Hirszfeld, [1], several reports have been published confirming this finding in a variety of human neoplasms as well as in spontaneous and experimentally induced animal tumors (for review see [2–4]). The carcinoembryonic antigen of the digestive system, (CEA), [5], and the α_1-fetoprotein, [6], were among the most thoroughly studied. However, the organ-system, and even the cancer-specificity, claimed originally for some of these carcinofetal antigens was disproved (for review see [3]). On the other hand, loss of normal gastrointestinal tissue antigens during carcinomatous transformation was observed [7, 8]. In addition, Davidsohn *et al.* [9, 10], Cowan [11], and Sheahan *et al.* [12], described the disappearance of BG-isoantigens in a considerable number of gastrointestinal (mostly gastric) neoplasms in contrast to Glynn *et al.* [13], Glynn and Holborow [14], and Eklund *et al.* [15].

The present communication deals with the behavior of BG in colonic carcinomas. BG-like activity was demonstrated by the mixed cell agglutination reaction, (MCAR) [16], as modified for tissue sections by Davidsohn and colleagues. (For review see [17])

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MATERIAL AND METHODS

Thirty-three colonic carcinomas, including 2 carcinomas of the cecum, (22 from patients with BG-A and 11 from patients with BG-B), and 8 metastases to the liver from colonic carcinomas were studied. Each bowel specimen contained both tumor and non-neoplastic normal mucosa. The liver metastases were surrounded by normal atrophic liver tissue. As additional non-malignant control tissue, 5 colonic polyps without histological signs of

malignancy were included. Histologically, the tumors were well to moderately well differentiated adenocarcinomas. The primary carcinomas, the polyps and the normal non-neoplastic colonic mucosa were obtained within 1–4 hr after surgery, and the liver metastases were autopsy material obtained up to 12 hr after the death of the patient. The material was fixed in neutral phosphate buffered formalin and embedded in paraffin by the usual routine techniques. MCAR was performed on 6 μm thick sections essentially as described by Kovarik *et al* [18]. By this method both water and alcohol soluble BG are detected. Briefly, deparaffinized tissue sections were washed with Tris–HCl buffer (pH 7·45) and subsequently covered and incubated with commercial BG-antisera (Ortho Diagnostics) for 15 min at room temperature in a moist chamber. The sera were concentrated prior to use to a titer in hemagglutination of 1:500 to 1:1000 and were used in this concentration. After incubation the slides were washed in 3 changes of Tris–HCl buffer (pH 7·45), covered with a 1% suspension of washed human isologous erythrocytes, and again incubated at room temperature for 30 min in the moist chamber. Thereafter the not specifically adherent erythrocytes were allowed to detach from the slide after inversion of the slide in buffer and the specimens were finally fixed by addition of glutaraldehyde to the buffer to a concentration of 1–5%. With this fixative, a good fixation of the specifically bound indicator red cells was achieved and the subsequent hematoxilin–eosin staining was possible without detachment of the cells. To assure the specificity of the MCAR, a number of controls was necessary: built-in positive controls were endothelial cells and erythrocytes, built-in negative controls were tissues known to be BG-negative like smooth muscle and connective tissue. Reagent controls included isologous antisera and non-isologous erythrocytes, and vice versa, as well as non-isologous antisera and non-isologous red cells. In additional control experiments, the BG-antisera were absorbed with the respective isologous erythrocytes prior to use.

Specimens from patients with BG-O were not included in our study since MCAR with Ulex europeus extract gave inconsistent results in our hands.

RESULTS

BG-like activity was present in an appreciable number of colonic carcinomas. (Table 1). In most of the tumors, the entire tumor area was BG-positive. Less often the BG-reactivity was confined to certain areas of the tumor whereas others were negative. Even superficially localized carcinomas bordering the lumen of the gut were BG-positive while the surrounding non-neoplastic mucosa did not express BG-like acitivity (Fig. 1). Normal non-neoplastic mucosa of the descending and the pelvic colon as well as of the rectum was invariably BG-negative contrary to the mucosa of the cecum which contained BG in its goblet cells. Adenomatous polyps of the colon without histologic evidence of malignancy did not contain BG (Fig. 2). From the 8 liver metastases derived from colonic cancers, histologically well to moderately well differentiated adenocarcinomas, 6 expressed BG-activity in uniform distribution, one in patchy distribution and one was negative (Fig. 3). Necrotic areas of positive carcinomas were BG-positive as well. Liver- and Kupffer-cells were BG-negative. Erythrocytes and endothelial cells showed positive MCAR in accordance with the erythrocyte blood group of the patient. BG non-compatible with the blood group of the patient were not seen under our experimental conditions. The positive MCAR was abolished by absorption of the BG-antisera with the respective isologous erythrocytes.

Table 1. Blood group substances (BG) in carcinomas of the distal large bowel of BG-A- and BG-B-patients (Number of tumors which contained BG-like activity, total number of cases studied are listed in parenthesis)

BG	A	B
Positive (total)	15 (22)	5 (11)
% Positives	68·2	45·4

DISCUSSION

Szulman [19] has shown that BG present in the epithelial cells of the fetal gut are rapidly fading from the distal segments of the large bowel during the neonatal period. The disappearance of BG was thought to be the result of degradation of these antigens by bacterial enzymes [20]. *In vitro* experiments proved indeed that BG are destroyed if incubated with intestinal bacteria [21, 22]. It is, however, questionable whether a similar mechanism is operable in the mucosa itself. The present results have shown that even superficially localized tumors which should be similarly accessible to bacterial enzymes as normal

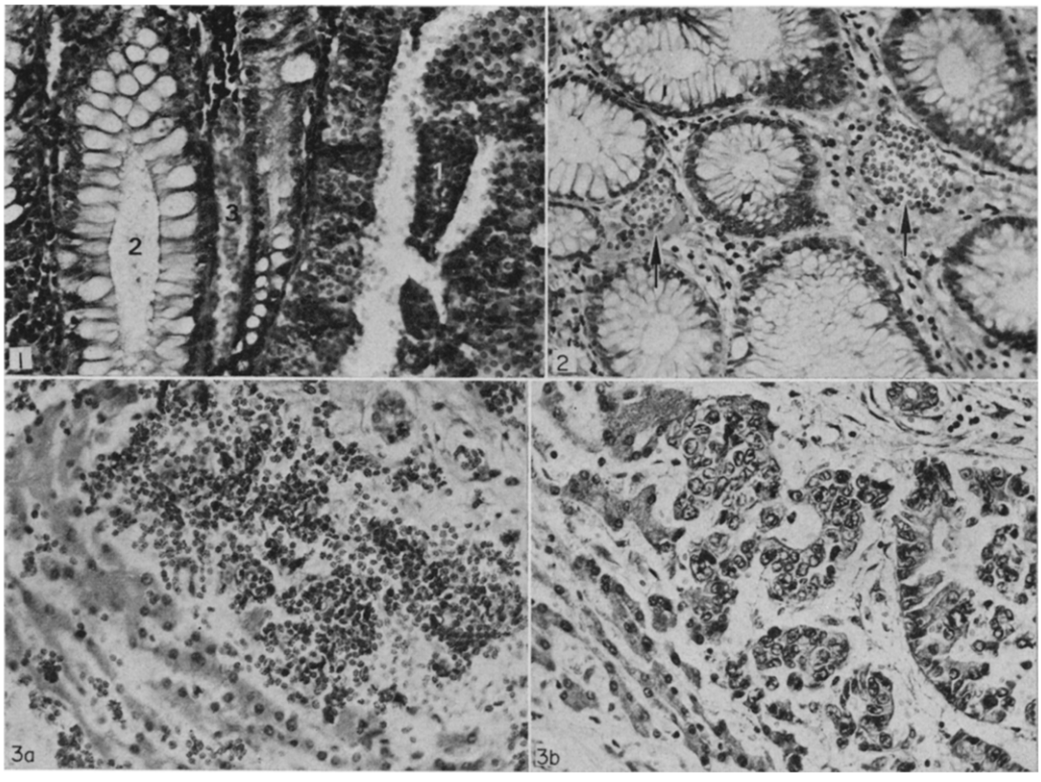


Fig. 1. *Carcinoma of the rectum of a patient with blood group A. Adherence of indicator erythrocytes to the carcinoma (positive MCAR)* (1), *negative non-neoplastic colonic mucosa* (2), *positive endothelium of a blood vessel (=built-in positive control)* (3), *Hematoxilin-Eosin* × 400.

Fig. 2. *Adenomatous colonic polyp without histologic evidence of malignancy of a patient with blood group A. Negative MCAR in the polyp, positive MCAR in endothelial cells and erythrocytes (arrow). Hematoxilin-Eosin* × 350.

Fig. 3. *A. Liver metastasis of colonic carcinoma of a patient with blood group A. Positive MCAR in the tumor after treatment of the section with BG-A-antiserum and BG-A-erythrocytes. Surrounding atrophic liver cells negative, positive MCAR with preexistent erythrocytes in the sinusoids. B. Consecutive section. Negative MCAR after treatment with BG-B-antiserum and BG-B-erythrocytes. Hematoxilin-Eosin* × 400.

(to face p. 488)

colonic mucosa, do contain BG. It seems likely, therefore, that repression of embryonic gene functions accounts for the disappearance of BG from the normal mucosa of the distal colon. Consequently, the reappearance of BG in carcinoms of the distal large bowel may represent genetic derepression in the tumor cells. From this point of view, normal and widely distributed antigens like BG can be considered as "carcinofetal antigens" if present in certain localizations, e.g. the distal large bowel. The possibility that BG-like substances from bowel bacteria are selectively absorbed by tumor cells as discussed by Cowan [11] is unlikely, particularly since metastases of colonic cancers to the liver were also BG-positive. The latter finding is somewhat surprising since Davidsohn and co-workers, (for review see [17]) and Sheahan *et al.* [12], have reported that BG disappear in most of the metastases of various carcinomas. Unfortunately, however, only one colonic carcinoma was included in the study of Sheahan *et al.* [12]. According to our results, metastatic lesions of colonic cancers seem to retain their BG in a higher percentage than those of other carcinomas. The localization of BG in primary and secondary colonic carcinomas differs from that previously described for CEA, [23–26]. It seems, therefore, that immunologic cross reactivity between BG and CEA observed with purified substances [27, 28] is not demonstrable in tissue sections with the morphologic method used. This point, however, is currently under investigation.

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REFERENCES

1. L. Hirszfeld, Untersuchungen über die serologischen Eigenschaften der Gewebe: über serologische Eigenschaften der Neubildungen. *Z. Immun.-Forsch.* **64,** 81 (1929).
2. P. Gold, Embryonic origin of human tumor-specific antigens. *Progr. exp. Tumor Res.* **14,** 43 (1971).
3. D. J. R. Laurence and A. Munro Neville, Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. *Brit. J. Cancer* **26,** 335 (1972).
4. E. H. Stonehill and A. Bendich, Retrogenetic expression: the reappearance of embryonal antigens in cancer cells. *Nature (Lond.)* **228,** 370 (1970).
5. P. Gold and S. O. Freedman, Specific carcinoembryonic antigens of the human digestive system. *J. exptl. Med.* **122,** 467 (1965).
6. G. I. Abelev, Production of embryonal serum globulins by hepatomas: review of experimental and clinical data. *Cancer Res.* **28,** 1344 (1968).
7. R. C. Nairn, G. E. Fothergill, M. G. McEntegard, H. G. Richmond, Loss of gastrointestinal-specific antigen in neoplasia. *Brit. med. J.* **I,** 1791 (1962).
8. P. Burtin, S. von Kleist and M. C. Sabine, Loss of a normal colonic membrane antigen in human cancers of the colon. *Cancer Res.* **31,** 1038 (1971).
9. I. Davidsohn, S. Kovarik and C. L. Lee, A, B, and O substances in gastrointestinal carcinoma. *Arch. Path.* **81,** 381 (1966).
10. I. Davidsohn, L. Y. Ni and R. Stejskal, Tissue isoantigens A, B, and H in carcinoma of the stomach *Arch. Path.* **92,** 456 (1971).
11. W. K. Cowan, Blood group antigens of human gastrointestinal carcinoma cells. *Brit. J. Cancer* **16,** 535 (1962).
12. D. G. Sheahan, S. A. Horowitz and N. Zamcheck, Deletion of epithelial ABH isoantigens in primary gastric neoplasms and in metastatic cancer. *Amer. J. dig. Dis.* **16,** 961 (1971).
13. L. E. Glynn, E. J. Holborow and G. D. Johnson, The distribution of blood-group substances in human gastric and duodenal mucosa. *Lancet* **ii,** 1083 (1957).
14. L. E. Glynn and E. J. Holborow, Distribution of blood-group substances in human tissues. *Brit. med. Bull.* **15,** 150 (1959).
15. A. E. Eklund, B. Gullbring and B. Lagerlöf, Blood group specific substances in human gastric carcinoma. A study using fluorescent antibody technique. *Acta path. microbiol. scand.* **59,** 447 (1966).
16. R. R. A. Coombs, D. Bedford and I. M. Ruillard, A and B blood group antigens on human epidermal cells demonstrated by mixed agglutination. *Lancet* **i,** 461 (1956).
17. I. Davidsohn, Early immunologic diagnosis and prognosis of carcinoma. *Amer. J. clin. Path.* **57,** 715 (1972).

18. S. Kovarik, I. Davidsohn and R. Stejskal, ABO antigens in cancer. Detection with the mixed cell agglutination reaction. *Arch. Path.* **86,** 12 (1968).
19. A. E. Szulman, The histological distribution of the blood group substances in man as disclosed by immunofluorescence. III. The A, B, and H antigens in embryos and fetuses from 18 mm in length. *J. exp. Med.* **119,** 503 (1964).
20. A. E. Szulman, The histological distribution of the blood group substances in man as disclosed by immunofluorescence. II. The H antigen and its relation to A and B antigens. *J. exp. Med.* **115,** 977 (1962).
21. L. Hoskins and N. Zamcheck, Bacterial degradation of gastrointestinal mucins. I. Comparison of mucus constituents in the stools of germ-free and conventional rats. *Gastroenterology* **54,** 210 (1968).
22. L. Hoskins, Bacterial degradation of gastrointestinal mucins. II. Bacterial origin of fetal ABH(O) blood group antigen-destroying enzymes. *Gastroenterology* **54,** 218 (1968).
23. S. von Kleist and P. Burtin, Localization cellulaire d'un antigène embryonnaire de tumeurs coliques humaines. *Int. J. Cancer* **4,** 874 (1969).
24. P. Burtin, S. von Kleist, M. C. Sabine and M. King, Immunohistological localization of carcinoembryonic antigen and nonspecific cross-reacting antigen in gastrointestinal normal and tumoral tissues. *Cancer Res.* **33,** 3299 (1973).
25. M. Bordes, R. Michiels and F. Martin, Detection by immunofluorescence of carcinoembryonic antigen in colonic carcinoma, other malignant or benign tumours, and non-cancerous tissues. *Digestion* **9,** 106 (1973).
26. H. Denk, G. Tappeiner, R. Eckerstorfer and J. H. Holzner, Carcinoembryonic antigen (CEA) in gastrointestinal and extragastrointestinal tumors and its relationship to tumor-cell differentiation. *Int. J. Cancer* **10,** 262 (1972).
27. C. Banjo, P. Gold, S. O. Freedman and J. Krupey, Immunologically active heterosaccharides of carcinoembryonic antigen of human digestive system. *Nature, New Biol. (Lond.)* **238,** 183 (1972).
28. M. D. Turner, T. A. Olivares, L. Harwell and M. S. Kleinman, Further purification of perchlorate-soluble antigen from human colonic carcinomata. *J. Immunol.* **108,** 1328 (1972).

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Immunization Against Gross Malignant Lymphoma with Mouse Cell Lines Chronically Infected with Non-Oncogenic Murine C-type Viruses

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Abstract—*Three* in vitro *mouse cell lines, chronically infected with non-oncogenic C-type viruses, were used for immunization of C57BL/6 mice against isograft of highly malignant Gross virus-induced lymphoma. The most effective immunizations were obtained with the 2 cell lines originating from C3H and BALB/c mice respectively, showing high degree of Gross virus-induced surface antigen. A third cell line, derived from a clone of L cells and similarily infected with a C-type virus, had no immunizing capacity. Protection of mice was obtained only after inoculation with the living cells. Cell-free culture supernatants were inefficient.*

SPONTANEOUS emergence of C-type viruses (CV) in longterm *in vitro* cultivated mouse cell lines was described by several authors [1–3]. More recently, pertinent data indicated that activation of CV can be obtained by the action of thymidine analogs: bromo- or iodo-deoxyuridine (BrdU or IodU) on mouse cells which were initially apparently virus-free [4]. A similar kind of activation was demonstrated following exposure of mouse cells to certain carcinogenic hydrocarbons [5]. These "endogenous" CV, appearing spontaneously or following inductive treatment, were proved to be in many if not all cases devoid of leukemogenic capacity when checked by inoculation of susceptible mice, even if a wide range of cell tropisms was covered in these assays. However, by their ultrastructural and antigenic characteristics these non-pathogenic CV were indistinguishable from the known pathogenic murine leukemia-sarcoma viruses [3, 6]. The biological or pathological potentialities and especially the possible epidemiological role of the CV in viral oncogenesis are, so far, not very well understood.

We reported previously [6] that the CV, released in cultures of two mouse cell lines were devoid of any leukemia producing capacity when inoculated into susceptible newborn C3HeB/Fe mice. However, these non-oncogenic CV, antigenically related to Gross leukemia virus (GLV), could interfere significantly in sensitive newborn mice of the same C3HeB/Fe strain with the leukemogenic process induced by a highly virulent GLV. Apparently no immunological mechanisms were involved in this interference phenomenon.

We are reporting here a quite significant preventive effect of non-pathogenic Gross type CV infected cultures on growth in mice of a malignant lymphoma, an effect which was apparently of an immunological nature.

MATERIAL AND METHODS

Mice

One to 2 months old, male C57BL/6 mice, were supplied by the Animal Selection Centre, Orléans, France.

Gross lymphoma (E♂G2)

Originally induced in a C57BL/6 mouse by

neonatal inoculation of Passage A GLV [7], this tumor line was obtained from Dr. Old (Sloan-Kettering Institute, New York, N.Y.) and maintained in our laboratory by intraperitoneal passages on male adult C57BL/6 mice. It was in its 178th to 199th transplant generation at the time of the here reported experiments. The E♂G2 cell suspensions for animal inoculation were prepared from enlarged spleens of leukemic mice: spleens were minced finely in PBS and allowed to stand for 1 minute to sediment larger fragments. The monodispersed cells in the supertanant fluid were centrifuged at 1000 rev/min for 10 min, and resuspended in PBS. Viable nucleated cells that excluded 0·4% trypan blue stain were counted. Intraperitoneal inoculation of adult C57BL/6 mice with 100 E♂G2 cells caused leukemic deaths in all inoculated mice within an average 17 days. The mice dead of leukemia showed at autopsy spleno-hepatomegaly and generalized adenopathy.

Cell lines

NCTC 2472 (N1) line, a clone isolated from a long-term culture of fibroblasts originating from the subcutaneous tissue of a normal C3H mouse [8], was provided by Dr. Sanford (NIH, Bethesda, Md.). The R4/B cell line, developed from the BALB/c3T3-clone 31 and transformed by radiation *in vitro* [9], was supplied by Dr. Todaro (National Cancer Institute, Bethesda, Md.). This line was adapted in our laboratory to grow in the presence of 30 μg/ml of BrdU. NCTC clone 929 (CL9) line, derived from the L cells of C3H mouse fibroblasts, was obtained from the American Type Culture Collection. EThV-A rat cell line [10], chronically infected with GLV, was supplied by Dr. Ioachim (Columbia University, N.Y.). The P4bisRR line, developed in our laboratory from lung tissue of a C57BL/6 mouse, was subsequently infected with a leukemogenic variant of Rauscher leukemic virus (RLV) [11].

All cultures were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% heated calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The *in vitro* passages were made weekly by trypsinization with a 0·25% trypsine solution.

Immunofluorescence tests

We applied the indirect method using living cell suspensions according to Möller's technique [12]. The type-specific rat anti-Gross serum, kindly supplied by Dr. Old, was obtained from (W/Fu × BN) F1 hybrid rats immunized against W/Fu rat leukemia (C58NT) D induced originally by wild type GLV from C58 mice [13]. The antiserum was absorbed once with RLV infected P4bisRR cells before use. The type-specific anti-Rauscher serum was prepared from BALB/c mice hyperimmunized with 6 intraperitoneal injections of *in vitro* cultured syngeneic T5 cells chronically infected with a low leukemogenic RLV. This serum was used after absorption with EThV-A cells carrying the GLV antigen. Fluoresceine-conjugated goat sera: (1) anti-rat gamma-globulin and (2) anti-mouse gamma-globulin were purchased from Hyland Laboratories (Los Angeles, California) and diluted with 2 volumes of PBS for the tests.

Tests with XC cells

In order to demonstrate the presence of infectious CV in cultures, direct XC tests, devised by Klement and Rowe [14], were used with some modifications. The XC cells were obtained from Dr. Rowe (NIH, Bethesda, Md.) and maintained in Eagle's MEM containing 10% fetal calf serum and 2 mM glutamine. The tests were performed as follows: 3×10^5 virus-carrier cells were seeded in flattened tubes containing 32×10 mm coverslips; The next day, when the cells had formed about 70% confluent cultures, 10^6 XC cells were overlaid. After cocultivation for 1–2 days, the coverslips were fixed with methanol and stained with Giemsa. The foci showing characteristic giant syncytia or tight groups of 3 or more smaller polycaryocytes were counted under the microscope. The number of foci was graded as follows: −; no foci, +; less than 10 foci, ++; 11 to 30 foci, +++; 31 to 70 foci, ++++; more than 71 foci.

Immunization

The 1–2 month-old male C57BL/6 mice received weekly intraperitoneal injections of 3×10^6 trypsinized cells of a line to be tested for its immunizing potential against the E♂G2 lymphoma. Control mice were injected similarly with the same number of spleen cells from normal C3H mice (except for Exp. No. I).

Inoculated mice were challanged, at the 7th day after last immunizing injection, by the same route, with usually 1000 E♂G2 cells (equivalent to 10LD100). The mice were observed by spleen palpation daily starting from the 10th day after challange. Number and survival time of mice dead with symptoms of leukemia were recorded.

Table 1. Characteristics of CV-infected cell lines used for immunization of C57BL/6 mice against challange with Gross E♂G2 lymphoma

Cell lines	Origin	Presence of CV at EM	IF* with sera		Direct XC test	Leukemogenic potential	Virus production particles/ml†
			anti-GLV	anti-RLV			
EThV-A	Rat (W/fu)	+	0·91	0·10	+ +	+ (Gross lymph. leuk.)	$5·9 \times 10^8$
N1	Mouse (C3H)	+	0·98	0·12	+ + + +	—	$5·6 \times 10^8$
R4/B	Mouse (BALB/c)	+	0·90	0·10	+ + + +	—	ND‡
CL9	Mouse (C3H)	+	0·50	0·32	—	—	$2·3 \times 10^8$
P4bisRR	Mouse (C57BL)	+	0·20	0·92	+ + + +	+ (Rauscher erythrobl. leukemia)	ND

*IF: Immunofluorescence index calculated after Möller' technique.

† : The numbers of virus particles per ml of culture media were measured by the Millipore filter counting method (Ref. 18).

‡ : ND: not done.

RESULTS

Characteristics of the five CV-infected culture lines used for experiments are summarized in Table 1. The presence of CV in these cultures was checked (1) by electron microscopy (EM) examination of either cells and ultracentrifugation pellets of cell-free culture supernatants, (2) by indirect immunofluorescent tests using cell suspensions and type-specific antisera (anti-Gross or anti-Rauscher) and (3) by direct XC test for polykaryocyte formations. In all 5 cell lines abundant CV, budding from the cell surface or present in the extra-cellular spaces and intracytoplasmic vacuols could be evidenced by EM examination. The 50 × concentrated CV suspensions, prepared from EThV-A or P4bisRR culture supernatants, produced respectively either Gross or Rauscher leukemia syndromes in susceptible C3HeB/Fe or BALB/c mice inoculated as new borns, whereas, in confirmation of previous data [6], the 50 × concentrated CV preparations from N1, R4/B, and CL9 cultures were entirely devoid of any leukemia producing capacity in both C3HeB/Fe and BALB/c newborn mice as concluded after at least 210 day's observation period. High fluorescent index (IF) (0·90–0·98) with type-specific anti-Gross serum and numerous formations of polycaryocytes in XC tests were regularly obtained with N1 and R4/B cells.

In preliminary experiments, we tried to preimmunize mice against fatal transplantable E♂G2 Gross lymphomas with the aid of heterologous EThV-A cells chronically infected with the leukemogenic GLV. Adult C57BL/6 males were inoculated intraperitoneally with 3×10^6 trypsinized cells or 1 ml of the cell-free culture supernatant, 6 times at weekly intervals. They were then challanged intraperitoneally, on the 7th day after last immunizing injection, with 1000 E♂G2 cells. As shown in Table 2 (Exp. No. 1), highly significant protection was observed in mice immunized with EThV-A cells as evidenced by a decreased mortality (56% survivors) and an increased survival time: average 24 days as compared with 15 days in the controls. Mice immunized with cell-free culture supernatant were nearly as susceptible to the challange as control mice.

In experiment No. 2 (Table 2), we compared the immunizing capacities of EThV-A cells with those of the non-leukemogenic CV-infected cells: R4/B, N1 and CL9. Mice which have received 3 weekly inoculations of 3×10^6 cells of either of these lines were challanged with 1000 E♂G2 cells. Control mice were injected similarly with the same number of spleen cells from normal C3HeB/Fe mice or with P4bisRR cells chronically infected with RLV. Higher protections were obtained in mice immunized with either R4/B cells (100%) or N1 cells (86%) than in those immunized with EThV-A cells (32%). All control mice, as well as those injected with CL9 cells, died with splenomegaly within 15 days after challange. In fact, single injection of 3×10^6 N1 cells confered a perfect protection of mice (close to 100%), not only against a challange with 1000 lymphoma cells (Exp. 3), but also against doses of these cells amounting to 3000 and even 6000 cells (Exp. 4).

In a parallel experiment, an attempt was made to immunize adult BALB/c mice by 6

Table 2. Immunization of C57BL/6 mice with in vitro *cell lines, chronically infected with leukemogenic or non-leukemogenic CV, against challange with malignant Gross E♂G2 lymphoma*

Exp.	Material for immunization	No. of inoc.‡	No. of E♂G2 for challange	No. of mice survived/ total*		Surv. time of leuk. mice averange	(range)
I	EThV-A cells	6×	1000	10/18	(56%)	24 days	(18–28)
	EThV-A supern.†	6×	1000	1/15	(7%)	16 days	(13–26)
	Control+	6×	1000	0/26	(0%)	15 days	(14–16)
II	EThV-A cells	3×	1000	6/19	(32%)	20 days	(15–22)
	N1 cells	3×	1000	19/22	(86%)	29 days	(15–30)
	N1 supern.	3×	1000	1/6	(17%)	20 days	(14–25)
	R4/B cells	3×	1000	10/10	(100%)		
	CL9 cells	3×	1000	0/14	(0%)	16 days	(15–18)
	P4bisRR cells	3×	1000	0/12	(0%)	15 days	(13–17)
	Control	3×	1000	0/23	(0%)	15 days	(13–16)
III	N1 cells	1×	1000	10/10	(100%)		
	Control	1×	1000	0/13	(0%)	14 days	(13–15)
IV	N1 cells	1×	1000	9/9	(100%)		
	Control	1×	1000	0/5	(0%)	15 days	(13–16)
	N1 cells	1×	3000	8/9	(89%)	30 days	
	Control	1×	3000	0/5	(0%)	13 days	(12–14)
	N1 cells	1×	6000	7/8	(87%)	25 days	
	Control	1×	6000	0/4	(0%)	14 days	(14)

+ Control mice inoculated with equivalent number of normal spleen cells.

†supern.: cell free culture supernatant.

‡3×10^6 trypsinized cells or 1 ml of cell free culture supernatants were inoculated intraperitoneally at weekly intervals. For controls, the same number of spleen cells of normal C3HeB/Fe mice (in Exp. 2, 3 and 4) 1 ml of fresh culture medium (in Exp. 1) were inoculated.

*Observation period: 3 months.

weekly intraperitoneal inoculations of 3×10^6 N1 cells against challange with virulent RLV; no protecting effect could be observed. The immunizing capacity of N1 cells was clearly virus type-specific and efficient exlusively against challange with Gross type murine leukemia cells.

No complement dependent cytotoxic antibodies against the E♂G2 cells could be evidenced in the sera of mice immunized with N1 cells. However, the inoculations of mice with 1000 E♂G2 cells mixed with 10^7 spleen cells taken from mice immunized with N1 cells, though it caused leukemia in all the inoculated animals, showed, nevertheless, a significant prolongation of survival times: 18 days in average as compared with 14 days in control mice which received the same numbers of E♂G2 admixed with normal spleen cells.

DISCUSSION

In confirmation of previously published data [3, 6] we found that 3 non-related mouse cell lines: N1, R4/B and CL9, originating respectively from C3H, BALB/c and C3H mice, were carrying a chronic infection with C-type viruses, antigenically identical or very close to the Gross-type leukemia virus but clearly different, according to the *in vitro* immunofluorescence test, from the FMR murine leukemia viruses. The origin of this chronic infections, whether exogeneous or, possibly, according to the recent data [4, 5], endogeneous, remains unknown. CV material collected from permanent cultures of these 3 lines was devoid of any leukemogenic capacity when inoculated in appropriate way into sensitive newborn mice. The mice used for these assays were of the C3HeB/Fe strain. Consequently, our experimentation using N1 and CL9 cells was done entirely inside the N-tropic system as defined by Hartley *et al.* [15].

Significant differences between the character of the CV infection in the CL9 and that of the N1 and R4/Bu cells, were noted though the amount of virus particles produced by the cells and accumulated in the culture medium was of the same order of magnitude for all the lines (2·3 to $5{\cdot}9 \times 10^8$ virus particles per ml); (1) the specific immunofluorescence index with anti-Gross leukemia serum was significantly lower for the CL9 than for the two other cell lines: 0·50 instead of 0·90 to 0·98; (2) furthermore, the XC test which may be considered as related to CV infectivity was constantly negative for the CL9 cells whereas it was highly positive for the N1 and R4/B cells.

These differences were paralleled by the negative results of vaccination with the CL9 cells against challange with the E♂G2 lymphoma, a vaccination which was quite efficient with the N1 and R4/B cells as well as with the heterologous EThV-A rat cells infected with exogeneous, leukemogenic GLV. The reasons underlying these differences are still unknown. The simplest, but probably unsufficient explanation would be that the specific Gross-type antigenicity of the CV from CL9 cells is lower as resulting from the mixed character of the CV population infecting this line. A weak but, nevertheless, significant index of immunofluorescence obtained with the CL9 cells exposed to anti-FMR serum is supporting this supposition. The alternative explanation would be related to lower infectivity of the CV from CL9 cells if compared with that of N1 and R4/B cells strains, as supported by the results of XC test. This is in concordance by the results obtained with the EThV-A cells which showed, equally, lower positivity with XC test and less prominent immunization efficiency. However, given the limited number of cell lines assayed, no definite conclusion can be drawn concerning the cause–effect relationship between production of infectious GLV particles by murine cell lines and their anti-Gross leukemia immunizing potential.

In a general way, in our present experiments, like in our former investigations on immunization against Rauscher leukemia [16, 17], with cell cultures chronically infected with non-pathogenic CV variants, the use for immunizing inoculations of living cells was much more efficient than the use of cell-free culture supernatants in which, still, massive presence of CV particles could be evidenced by electron microscopy. It is quite possible that living homologous cells continuing, for few days after inoculation, their synthetic activity, not only produce more viral material but also supply, as one can suppose, in addition, some specific, virus-induced cell antigens playing a role in the active immunization against the leukemic process.

It can be concluded that variants of CV having Gross murine leukemia type antigenicity can not only interfere, when introduced into newborn mice, with the initiation of the leukemic process, as previously demonstrated [6], they can also, when inoculated into the animal at a proper time under the form of virus-infected cells, hamper the replication of "ready" leukemic cells by triggering a specific immune reaction directed against these cells. This is at least so in the case of C57BL mice in which an overt Gross-type C-virus infection is normally not observed and which, consequently, do not develop at the perinatal period immuno-tolerance to this infection.

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REFERENCES

1. S. Dales and A. F. Howatson, Virus-like particles in association with *L* strain cells. *Cancer Res.* **21,** 193 (1961).
2. D. A. Kindig and W. H. Kirsten, Virus-like particles in established murine cell lines; electron microscopic observations. *Science* (*N.Y.*) **155,** 1543 (1967).
3. W. T. Hall, W. F. Andersen and V. J. Evans, *In vitro* and *in vivo* observation on a murine C-type virus. *J. Virol.* **1,** 1238 (1967).
4. S. A. Aaronson, G. J. Todaro and E. M. Scolnick, Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. *Science* (*N.Y.*) **174,** 157 (1971).
5. D. Barbieri, J. Jr. Belehradek and G. Barski, Decrease in tumor-producing capacity of mouse cell lines following infection with mouse leukemia viruses. *Int. J. Cancer* **7,** 364 (1971).
6. G. Barski and J. K. Youn, Protection of mice against Gross leukemia by interfering action of non-leukemogenic C-type murine viruses inoculated into newborns. *J. nat. Cancer Inst.* **47,** 575 (1971).
7. L. J. Old, E. A. Boyse and E. Stockert, The G (Gross) leukemia antigen. *Cancer Res.* **25,** 813 (1965).
8. K. K. Sanford, R. M. Merwin, G. L. Hobbs, J. M. Young and W. R. Earle, Clonal analysis of variant cell lines transformed to malignant cells in tissue culture. *J. nat. Cancer Inst.* **23,** 1035 (1959).
9. E. J. Pollock, S. A. Aaronson and G. J. Todaro, X-irradiation of BALB/3T3. Sarcoma-forming ability and virus induction. *Int. J. Radiat. Biol.* **17,** 97 (1970).

10. H. L. Ioachim, Long-term replication of Gross leukemia virus (GLV) in thymic cultures. *Proc. Amer. Assoc. Cancer Res.* **7,** 33 (1966).
11. D. Barbieri, G. Barski and J. K. Youn, Variantes du virus de Rauscher en cultures de cellules d'origine pulmonaire de la souris C57BL. *C.r. Acad. Sci. (Paris)* **256,** 2145 (1967).
12. G. Möller, Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. *J. exp. Med.* **114,** 415 (1961).
13. L. J. Old, E. Stockert, E. A. Boyse and G. Geering, A study of passive immunization against a transplanted G leukemia with specific antiserum. *Proc. Soc. exp. Biol.* **124,** 63 (1967)...
14. V. Klement, W. P. Rowe, J. W. Hartley and W. E. Pugh, Mixed culture cytopathogenicity. A new test for growth of murine leukemia viruses in tissue culture. *Proc. nat. Acad. Sci.* **63,** 753 (1969).
15. J. W. Hartley, W. P. Rowe and R. J. Huebner, Host-range restrictions of murine leukemia viruses in mouse embryo cell cultures. *J. Virol.* **5,** 221 (1970).
16. G. Barski and J. K. Youn, Immunization against Rauscher mouse leukemia with tissue culture material. *Science (N.Y.)* **149,** 751 (1965).
17. J. K. Youn, G. Barski and D. Barbieri, Mécanisme d'immunisation préventive à l'aide de cultures de virus de Rauscher à pouvoir leucémigène atténué. *Int. J. Cancer* **3,** 116 (1968).
18. D. Barbieri, E. Delain, P. Lazar, G. Hue et G. Barski, Method of virus particles counting using Millipore filtration. *Virology* **42,** 544 (1970).

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A Clinical Study of the Natural History of Lymphosarcoma and Reticulum Cell Sarcoma

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Abstract—*A retrospective review of* 182 *previously untreated cases of lymphosarcoma (LSA) and reticulum cell sarcoma (RCS) seen during a thirty month period suggests four distinct patterns of initial disease presentation with important prognostic features.* 1. *A localized form of disease presenting at a single node region or an extranodal site, had the longest survival. The median survival of the nodal group was* 56 *months for LSA and* 33 *months for RCS, while the extranodal group had median survival of* 48 *months for LSA and* 38 *months for RCS.* 2. *A loco-regional form of disease presenting at nodal and extranodal site(s) strictly confined within the abdominal cavity, had a median survival of* 16 *months for LSA and* 12 *months for RCS. Approximately half of these patients presented as diffuse disease involving multiple nodal and/or extranodal sites. This diffuse* intra-*abdominal pattern appears to be a recognizable entity and seems uniquely suitable for total abdominal irradiation. An extra-abdominal form of loco-regional disease involving an extranodal site along with the corresponding node region was also seen but relatively infrequently, and initial disease presentation at* 2 *or more node regions on one side of the diaphragm was rarely seen.* 3. *A generalized nodal pattern of presentations at node regions both above and below the diaphragm was fairly frequently observed* (40% *in LSA and* 22% *in RCS). The median survival of this group was* 35 *months for LSA and* 23 *months for RCS.* 4. *A disseminated form of disease involving multiple nodal and extranodal site(s) seen in over one-third of the cases, had a very poor survival* (7 *months for LSA and* 6 *months for RCS).*

No satisfactory clinical staging system for LSA and RCS is available. Taking the above mentioned patterns of disease presentation in consideration, an anatomic clinical staging system for LSA and RCS is under study at Memorial Hospital. Before an appropriate staging system is available, recognition of the different patterns of disease presentation bearing clear prognostic and therapeutic implications, will help in the total management of non-Hodgkin's lymphomas.

INTRODUCTION

THE INTRODUCTION of clinical staging system for Hodgkin's Disease (HD) has helped to define its natural history and contributed significantly to its clinical management. Since lymphosarcoma (LSA) and reticulum cell sarcoma (RCS) are often included with HD, under the broad term "lymphoma", the Rye classification for Hodgkin's Disease had been employed for staging LSA and RCS. A staging system designed for HD, however, denies recognition of significant differences in the biologic behaviors of LSA and RCS [1–10] and ignores certain characteristic patterns of clinical presentation which bear important prognostic and therapeutic implications.

Over the last decade, introduction of sophisticated diagnostic methods have considerably extended the scope of evaluating the extent of lymphomatous disease. At Memorial Hospital since the mid-sixties these procedures have been regularly employed in the evaluation of extent of disease, and a large number of such

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cases with LSA and RCS has been gathered. The following retrospective study was undertaken in an attempt to delineate the natural history and the biologic behaviour of LSA and RCS. A better understanding of the natural history might indicate a staging system more appropriate to these non-Hodgkin's lymphomas.

MATERIAL AND METHODS

The study included a retrospective review of the clinical records of all adult patients over the age of 15 with LSA and RCS seen at Memorial Hospital between January 1966 and June 1969. The only patients excluded from the analysis were those who received therapy at an outside facility prior to being seen at Memorial Hospital. The purpose of this exclusion was to maintain uniformity and objectivity in patient work-up prior to therapy for the purposes of determining the mode of anatomic presentation. This particular time period was chosen for two reasons: 1. to allow a two and one-half year survival analysis and 2. to evaluate a reasonably standard patient work-up, as most modern investigative procedures such as lymphangiography, isotope scanning and other sophisticated methods have been regularly employed in evaluation of extent of disease since the beginning of 1966.

Criteria for evaluation of extent of disease

In order to assure an objective appraisal of the extent of disease, the following criteria were used; 1. All radiologically positive lymphangiograms and any concurrent or subsequent adenopathy were considered as evidence of disease involvement unless proven otherwise. 2. Splenomegaly documented by physical examination or by isotope scanning was considered positive. 3. Hepatic involvement was accepted when any of the following criteria was available; (*a*) positive liver biopsy, (*b*) positive isotope scan of the liver, (*c*) abnormal liver function tests (in absence of any other hepatic disease), (*d*) unequivocal hepatic enlargement in absence of other non-neoplastic causes. 4. Pericardial, cardiac, skin and soft tissue involvements were accepted only if biopsy confirmed the presence of lymphoma. 5. Pulmonary involvement was recognized from biopsy, cytology or appropriate radiographic findings; pleural effusion and ascites were accepted as evidence of disease only in absence of other non-neoplastic causes. 6. Gastro-intestinal, renal, and bone involvement were accepted only from biopsy or appropriate radiographic studies. Neurological involvement was considered only from positive spinal fluid cytology or appropriate radiographic or isotope scanning evidence.

RESULTS

One hundred eighty-eight cases (76 with LSA and 112 with RCS) fulfilled our criteria for inclusion in this study.

Age and sex distribution

Very few children with LSA and RCS were seen during this period who could qualify for analysis. Therefore, we have excluded the childhood cases from our analysis. The age distribution is shown in Fig. 1. The Male: Female ratio in LSA was 2:1, and in RCS 1·7:1.

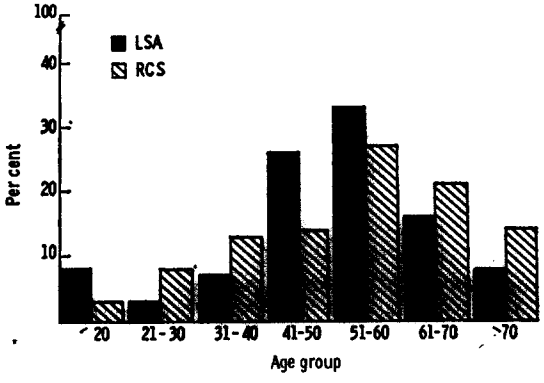


Fig. 1. *Incidence of lymphosarcoma and reticulum cell sarcoma by age.*

Initial work-up

All patients had a complete history and physical examination, hemogram and chest X-rays. Liver function studies were available in 150 out of 188 cases (80%). Lymphangiography was not done in the presence of palpable or otherwise demonstrable disease below the diaphragm or in the presence of diffuse extranodal involvement. Similarly, laparotomies were done for specific indication only (Table 1). The total number of cases undergoing lymphangiography or laparotomy was 52 in LSA (68·5%) and 85 in RCS (76·1%), a total of 137 cases (72%).

Site(s) of disease involvement at diagnosis

At diagnosis, disease was found to be confined within the lymphatic architecture in 41 of 76 cases (54%) with LSA, and only 32 of 112 cases with RCS (28·5%). The others were found to have disease at extranodal site(s) with or without nodal involvement at diagnosis. Disseminated disease involving multiple nodal and extranodal site(s) was found in 30% of cases with LSA and 43% of cases with RCS. Retro-

Table 1. Evidence of retroperitoneal node involvement by lymphangiography (LAG) or laparotomy (LAP) in patients with lymphosarcoma (LSA) and reticulum cell sarcoma (RCS)

	No. LAG	Positive LAG No.	%	No. LAP	Positive retroperitoneal node No.	%	Per cent positive retroperitoneal Node by LAG/LAP
LSA	31	21	68	21	15	71	69
RCS	58	41	71	27	17	63	68

peritoneal node involvement was found in thirty-six (69%) of fifty-two cases with LSA and fifty-eight (68%) of eighty-five cases with RCS who underwent lymphangiography or laparotomy (Table 1).

The most frequently involved extralymphatic sites were; liver, gastro-intestinal tract, skin and subcutaneous tissue, bone, lung and mesentery. Pleural effusions and ascites were noted frequently. The relative incidence of these has been shown in Fig. 2.

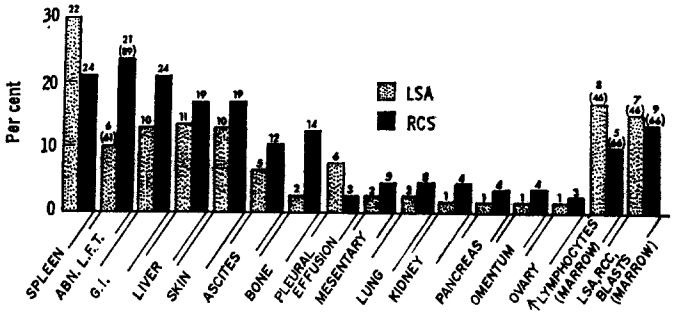


Fig. 2. Incidence of extra-nodal involvement by lymphosarcoma and reticulum cell sarcoma at diagnosis. The relative incidence of bone marrow involvement has been calculated from the actual number of bone marrow examinations performed. Number in each box represents the number of cases, and the number in parenthesis represents the number of tests or procedures done.

Other less frequently involved sites were pancreas, omentum, adrenals, ovary, CNS, peritoneum, uterus, cervix, gingiva, and pericardium. Superior vena cava syndrome was found in 4·5% of the cases with RCS but none in LSA. Forty-six of seventy-six cases with LSA and sixty-six of one hundred twelve cases with RCS had bone marrow examination. An increase in lymphocyte count was found in eight cases with LSA (17%) and five cases with RCS (7·5%). Immature cells with cleft nuclei (LSA cells), RCS cells, or with blastoid features were found in seven cases (15%) with LSA and nine (13·5%) cases with RCS (Fig. 2).

Systemic symptoms (fever, sweats, weight loss exceeding 10% of the body weight) were recorded in eight cases with LSA (10%) and twenty-one cases with RCS (18·5%). With the exception of one case with LSA and two cases with RCS, all patients recorded as having systemic symptoms had widespread disease.

Mode of initial disease presentation

Four major patterns of anatomic presentation became apparent (Table 2).

1. A localized form of presentation involving either a single node region or a single extranodal site was found in 15·5% of patients with LSA (11·5% nodal and 4% extranodal) and 16% with RCS (5% nodal and 11% extranodal).

2. A locoregional presentation at extranodal site(s) with or without involvement of node regions, strictly confined within the abdominal cavity was noted in 9% of the cases with LSA and 11·5% of the cases with RCS. Approximately half of these cases presented as diffuse intraabdominal disease involving multiple nodal and/or extranodal site(s).

3. A generalized nodal form of disease presenting at node regions both above and below the diaphragm was found in 40% of the cases with LSA and 22% with RCS.

4. A disseminated form of disease involving multiple nodal and/or extranodal site(s) was also frequently encountered (30% in LSA and 43·5% in RCS). The disseminated form of presentation was more frequently found in RCS than in LSA and was twice as frequent as the generalized nodal form of presentation.

Another loco-regional presentation involving an extranodal site along with the corresponding node region outside the abdominal cavity was

Table 2. Patterns of clinical–anatomic presentation and its relationship to lymphosarcoma (LSA) and reticulum cell sarcoma (RCS)

	LSA		RCS		Median survival in months	
	No.	%	No.	%	LSA	RCS
Localized — Nodal	9	11·5	6	5·0	56	33
Localized — Extra-nodal	3	4·0	12	11·0	45+,48+,55+*	38
Loco-regional — Intra-abdominal	7	9·0	13	11·5	16	12
Loco-regional — Extra-abdominal	2	3·0	6	5·0	18,32+*	18
Generalized Nodal	30	40·0	24	22·0	35	23
Disseminated	23	30·0	49	43·5	7	6

* There were too few cases to derive a median survival; instead, the actual survival of the individual cases are shown.

The median survival figures are calculated by plotting the individual survival data. Because of a relatively large number of cases seen in 1966 and 1967, the median survival figures have been longer in localized and generalized nodal groups.

seen at times (3% in LSA and 5% RCS). Presentation of disease at 2 or more node regions at one side of the diaphragm was rarely encountered (only 4 cases seen in this study).

Mode of progression and recurrence

Of the thirty-four cases with localized disease, adequate follow-up information was available in thirty cases to determine the first site(s) of progression. (Three cases were lost and mode of progression could not be determined in one case because of early death due to post-operative complication of diagnostic laparotomy). Of these thirty cases, eighteen cases continue to live clinically free of disease as of December 1971. Clinical evidence of progression of disease was noted only in twelve cases showing progression of disease, evidence of spread from one side of the diaphragm to the other side was found in two cases with LSA. In both of these, initial disease was supra-diaphragmatic and subsequent involvement was below the diaphragm, however, neither of these cases had initial lymphangiography.

The only other progression noted in the LSA group was on the same side of the diaphragm, in this case the disease progressed forty-four months after diagnosis from right axilla to left axilla which had not been included in the original radiation port. In the RCS group, progression occurred in nine cases, and in all of these it was noted on the same side of the diaphragm. Two of these nine cases had local recurrence and the others usually progressed to the contiguous or other node bearing areas. In the generalized nodal group, the only consistent pattern of progression that could be determined was that in eleven of twenty-six adequately followed cases with LSA and seven of fourteen with RCS, the first site of progression was within the nodal architecture. The median time to progression of disease was thirteen months in LSA and eight months in RCS. Progression to extranodal sites both above and below the diaphragm, was frequently noted and such incidence was more frequent and widespread in RCS.

Therapy

We have analyzed the initial therapy and its effect upon survival without attempting to analyze the subsequent therapy during the course of disease. Most patients with localized disease were treated with surgery (either node dissection or wide excision of extra-nodal disease bearing site), or radiation therapy alone either to a limited portal involving only the disease bearing area or to an extended field, including the disease site and the contiguous node bearing areas. Total nodal irradiation was not used in this group. Some patients with localized disease were treated with a combination of surgery and radiotherapy or chemotherapy. The dose of radiotherapy varied from 1250 rads to 6200 rads tumor dose (average 3200 rads).

Therapy for patients with intra-abdominal disease usually consisted of either surgery followed by irradiation, local abdominal irradiation or irradiation to the para-aortic and inguinal node bearing areas. Some form of chemotherapy was added to the initial treatment in only three patients.

In the generalized nodal group, regional or local radiotherapy was the single most common modality of therapy and was often preceded or

followed by chemotherapy. Radical radiotherapy, in the form of total nodal radiation was administered to only three patients as initial treatment. Ten patients (nine with LSA and one with RCS) received only chemotherapy as the primary treatment. No appreciable difference was found in survival, or in the interval to progression in patients receiving chemotherapy alone or radiation and chemotherapy. Most patients with disseminated disease involving multiple nodal and extranodal sites received chemotherapy with or without local radiotherapy. The most commonly used single or combination chemotherapeutic agents included vincristine, chlorambucil, cyclophosphamide, and prednisone.

Survival

One hundred seventy-one cases (71 with LSA and 100 with RCS) were available for survival analysis. Eight cases were lost to follow-up and nine cases were excluded from survival analysis because of death from unrelated causes (myocardial infarction, pulmonary embolism, CVA, etc.). The survival for seven cases with giant follicular lymphosarcoma was separately done and all of them were alive at the time of analysis. Table 2 summarizes the median survival of each group. No appreciable difference in median survival could be found between localized nodal and extranodal disease in LSA or RCS.

In the LSA group, the median survival for localized nodal disease was 56 months and all three cases with localized extranodal disease are surviving for 45+, 48+, and 55+ months.

The median survival for the loco-regional abdominal group in LSA and RCS was 16 months and 12 months respectively. The median survival figure for the loco-regional extraabdominal group could not be derived. The survival of individual cases in LSA were 18 and 32+ months and in RCS were, 6,11,18, 21, and 39+ months. Three of four cases (LSA and RCS) with disease presenting at two or more node regions on one side of the diaphragm survived for 34+, 55 and 61+ months, the follow-up of one case being lost.

The median survival of the generalized nodal group in LSA and RCS was 35 months and 23 months respectively. In sharp contrast the median survival for the disseminated disease group in LSA was 7 months and in RCS 6 months.

Figures 3 and 4 show the two-and-one-half year survival analysis. All cases with localized disease (nodal and extranodal) in LSA were alive at the time of analysis and 82% of the

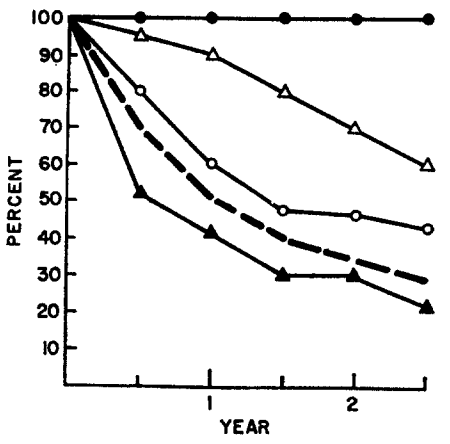


Fig. 3. Two and a half year survival of individual groups of anatomic disease presentation in lymphosarcoma. (●——● localized, ○——○ loco-regional intra-abdominal, △——△ generalized nodal, ▲——▲ disseminated disease group). The heavy dotted line represents the survival curve of cases that would have been staged as Stage IV (Rye).

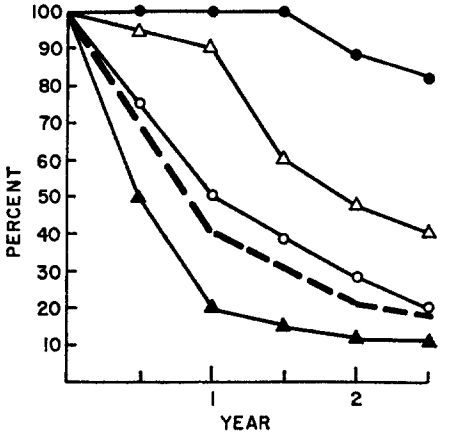


Fig. 4. Two and a half year survival of individual groups of anatomic disease presentation in reticulum cell sarcoma. (●——● localized, ○——○ loco-regional intra-abdominal, △——△ generalized nodal, ▲——▲ disseminated disease group). The heavy dotted line represents the survival curve of cases that would have been staged as Stage IV (Rye).

cases in the corresponding group with RCS were alive at two-and-one-half years. A marked difference was found for the intraabdominal group at two and one half years with only 43% in LSA and 20% in RCS surviving. In the generalized nodal group, the corresponding survival figure in LSA and RCS was 60% and 40% while in the disseminated group it was 22% for LSA and 11% for RCS. If survival data for these cases, which would have been considered at Stage IV (Rye) were plotted, the two-and-one-half year survival would be 29% in LSA and 18% in RCS.

Prognostic features

No correlation could be found between survival and age in this study. The prognosis

of individual organ involvement has not been analyzed. However, a correlation between survival and systemic symptoms, hemoglobin levels, and none marrow findings (with autopsy data) has been made.

In the LSA group, three of eight cases described as having systemic symptoms, had only nodal disease and they survived for 56+, 61+, and 15 months correspondingly. The remaining five cases had widespread disease and survived for 4, 5, 5, 14 and 53 months. In the RCS group four of twenty-one cases were found to have only nodal disease and they survived for 34, 16, 36, and 5 months. The remaining seventeen cases were found to have rather extensive disease and the median survival of this group was 4 months (range 1–32 months).

In the LSA group, twenty cases were found to have hemoglobin levels below 12 g% and their median survival was 15 months (range 1–67+ months). The median survival of the remaining cases with hemoglobin levels over 12 g% was 36 months (range 1–69+ months). In the RCS group, thirty cases had hemoglobin levels below 12 g% and their median survival was 7 months (range 1–60+ months). The remaining cases with hemoglobin levels over 12 g%, had a median survival of 20 months (range 1–60+ months). In both the LSA and RCS groups, only five of fifty cases with hemoglobin levels below 12 g% had bone marrow involvement and those five cases survived for 1, 2, 7, 7, and 14 months.

Table 3 summarizes the correlation between survival and results of the bone marrow examinations. In the LSA group, only one of eight patients who showed an increase in the lymphocytes in the marrow, was found to have documented marrow involvement with LSA cells at autopsy. Four cases in this group are still alive from 35+ to 58+ months. All five cases showing LSA cells or immature cells with blastoid features, were found to have bone marrow involvement at autopsy and they survived for 3, 7, 14, 19, and 40 months. In the RCS group, three out of five cases having an increase in the lymphocytes in the bone marrow died, and only one was found to have bone marrow involvement at autopsy. The autopsy of the other two cases were negative for marrow involvement. All five cases, excluding one whose follow-up was lost, survived for 17, 24, 30+, and 35 months. (The only case showing marrow involvement at autopsy survived for 24 months). Eight out of the nine cases diagnosed as having either RCS cells or immature cells with blastoid features are already dead. Six cases had autopsies, and all of them were found to have marrow involvement. The median survival of this group was 3 months (range 1–32 months). The two cases who did not have an autopsy survived for 7 and 13 months (Table 5.). The follow-up in one case was lost.

DISCUSSION

Several studies [1–2,4–8,10,11] have been reported in the literature dealing with the natural history of LSA and RCS. Though there have been differences in results of such studies, it has been generally agreed that LSA and particularly, RCS, present in extralymphatic sites more frequently than does HD [1,4,12]. Freeman, *et al.*, [13] have recently reviewed the incidence of extralymphatic origin in LSA and RCS from cases collected by the end result group of cancer registry and found the incidence of extralymphatic origin in LSA and RCS to be 15% and 24% respectively. Others have reported even higher figures [5,6,12]. In our study, the relatively high incidence of extralymphatic involvement may be partly due to more extensive disease work-up including laparotomy which was done in 26% of the cases. Evidence is now available that laparot-

Table 3. The correlation between bone marrow findings, survival and post mortem examinations in patients with lymphosarcoma (LSA) and reticulum cell sarcoma (RCS)

Diagnosis	Bone marrow findings	Survival	Post mortem bone marrow examinations No. Done	No. Positive	Comment
LSA	Increased lymphocytes	15,35+,45+,48,56+,58+,L,L*	1	1	Four still living.
LSA	LSA cells	3,7,19,40	4	4	
LSA	Increased blasts	14	1	1	Died in a leukemic phase.
RCS	Increased lymphocytes	17,24,30+,35,L.	3	1	One still living.
RCS	RCS cells	1,2,3,7,13,32	4	4	No autopsies in two cases.
RCS	Increased blasts	1,1,L	2	2	

*L = Lost to follow-up.

omy does reveal occult disease in LSA and RCS. [14] which would have been undetected otherwise. Detection of involvement of occult intra-abdominal sites such as pancreas, omentum, mesentery, ovary, adrenals, liver, etc., is not always possible without laparotomy. It is noteworthy that there is a significant number of cases with involvement of such occult sites in this series, (Fig. 2). It may partly, also, be due to a selective referral of patients with relatively advanced forms of the disease to Memorial Hospital. The figure for the bone marrow involvement in this series, has been derived from the total number of patients who had had bone marrow examination. We have not considered the presence of an increase in lymphocyte count in the bone marrow as evidence of disease involvement, because of the fact that only two of thirteen such cases showing an increase in lymphocytes in the marrow were found to have documented marrow invasion at autopsy or subsequent examination and such increase in lymphocyte count in marrow did not seem to adversely affect survival (Table 3).

Comparison of the incidence of retroperitoneal node involvement in this series with that in other reports is not possible, as the major reports in the literature [2,4,8] include patients seen prior to 1960; since lymphangiography was not introduced to regular clinical medicine before the early 1960's, such comparison would be unrealistic. It is, however, well documented that lymphangiography does reveal evidence of retroperitoneal node involvement in a relatively high percentage of cases with LSA and RCS, irrespective of stage [3]. Several studies have been reported in the literature dealing with staging and survival analysis. Rosenberg *et al.*, [7,8] found no difference in two and five year survival in their Stage II and III group. As a matter of fact, the two year median survival in Stage III in LSA (52%) was slightly better than that of Stage II (44·9%). Since none of Rosenberg's cases had lymphangiography, this may be explained due to the fact that perhaps, many of his Stage II's might have indeed been Stage III's. This would also explain the relatively high number of Stage II's in his series.

It is noteworthy that cases which would have been considered as Stage IV (Rye Classification) did poorly. However, when the survival data of the cases with localized single organ involvement were separately plotted, the superiority in prognosis of the localized extranodal group became clearly appreciable (Figs. 3 and 4).

Several major patterns emerge from this study. 1. Localized extralymphatic site involvement bears the same prognosis as that of disease involving one node bearing area only, (Table 2). 2. The incidence of disease presentation at two or more node bearing areas on one side of the diaphragm was very low in this series. This is in full agreement with our earlier experience [3] with lymphangiography which revealed that the incidence of retroperitoneal node involvement ranges from 80–100% in LSA and RCS, irrespective of pre-lymphangiography stages. 3. Though their number is low, yet a diffuse form of intra-abdominal disease involving several extralymphatic sites, with or without nodal involvement, appears as a recognizable entity. 4. The prognosis of the loco-regional intra-abdominal disease is distinctly poorer than that of the generalized nodal group (Figs. 3 and 4), and the survival curve in the loco-regional group almost parallels that of the disseminated group. 5. Despite the conventional belief that generalized nodal involvement bears a very poor prognosis in these neoplasms, our study revealed that the prognosis of this group is relatively better (median survival is 35 months in LSA and 23 months in RCS) than usually thought. 6. The prognosis of the disseminated group, which constitutes a major proportion (30% in LSA and 41% in RCS) is indeed very poor.

Two important prognostic criteria in this study appeared to be; 1. the extent of disease and 2. the hemoglobin level. Cases with hemoglobin levels under 12 g% irrespective of bone marrow involvement did considerably poorer than those with hemoglobin levels over 12 g%. The other prognostic feature of clinical importance was the bone marrow findings. Increase in lymphocyte count in the marrow did not adversely affect the survival, whereas bone marrow invasion with immature cells with cleft nuclei (LSA cells), RCS cells, or cells with blastic features usually signified poor prognosis.

The recognition of the intra-abdominal presentation of non-Hodgkin's lymphoma may seem to identify that group of patients who may benefit uniquely from total abdominal irradiation.

Systemic symptoms were noted in overwhelming majority in disseminated disease and they did not seem to influence the naturally poor prognosis of this group. No histological correlation with survival has been attempted though this study reaffirms the better prognosis of giant follicular lymphosarcoma.

There is no clear understanding regarding the pattern of spread of these two neoplasms.

Musshoff *et al.* [4] maintain that after a disease free interval, recurrences in distant lymph nodes were four times more frequent in cases with primary lymph node involvement than in cases with primary organ involvement (contrary to ours, they however, found that incidence of primary extralymphatic origin is relatively rare). Our study, though not conclusive, reveals that in localized disease, the majority of initial progressions were confined to the same side of the diaphragm and nodal progressions were more frequent than progression to extranodal sites, irrespective of origin.

Lymphangiography still remains one of the most valuable diagnostic tools to examine the lymphatic structures in retroperitoneal space. Since incidence of retroperitoneal node involvement is very high, it is desirable that bilateral pedal lymphangiography should be a definite part of pretherapy patient work-up. In our study, we have encountered two incidences of false negative lymphangiogram, and since there is some evidence of the possibility of picking up occult sites by laparotomy, [14] it appears then, that laparotomies would contribute to pretherapy disease evaluation, particularly in the generalized nodal group. A prospective study with more cases will be required to answer this question more definitely.

No satisfactory clinical staging system for LSA and RCS is currently available. The "Rye Classification" [15] for staging Hodgkin's disease (HD) has been used for staging LSA and RCS by many, and more recently an improved anatomic staging classification [16] has been proposed by the committee on Hodgkin's disease staging classification at Ann Arbor. A clinical staging system for HD, however, does not seem to be applicable for staging purposes of LSA and RCS since the biologic behavior of these neoplasms appear to differ from that of HD: 1. In contrast to HD, the incidence of LSA and RCS presenting at two node bearing areas on one side of the diaphragm is extremely low, though a somewhat advanced form of disease (involving extranodal site(s) with or without nodal involvement) strictly limited to the abdominal cavity appears to be a recognizable entity. 2. These neoplasms often present as a generalized process and of the generalized forms of disease the generalized nodal form appears to have a better prognosis. 3. The relatively higher incidence of extralymphatic involvement in LSA and RCS at the time of the diagnosis, make them quite different from HD in their mode of presentation [1,4,12]. 4. Prognosis of the loco-regional form of disease is distinctly poorer than that of the strictly localized extranodal form. Though LSA and RCS are often grouped together with HD under the broad term "lymphoma", it is desirable that the above mentioned features of these neoplasms be considered in their clinical staging. A staging system considering these features for LSA and RCS would be extremely useful for their management, prognostic determination, and exchange of information. Taking these into consideration a new clinical staging system for LSA and RCS is currently under study at Memorial Hospital. Before such a system is available, recognition of the different patterns of anatomic presentation of LSA and RCS will be useful for the total management of these two neoplasms.

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REFERENCES

1. A. Banfi, G. Bonadonna, G. Carnevali, C. Oldini and E. Salvini, Preferential sites of involvement and spread in malignant lymphomas. *Europ. J. Cancer* **4,** 319 (1968).
2. G. Hilton and P. M. Sutton, Malignant lymphomas: classification, prognosis, and treatment. *The Lancet* **i,** 284 (1962).
3. B. J. Lee, J. H. Nelson and G. Schwarz, Evaluation of lymphangiography, inferior venacavagraphy, and intravenous pyelography in the clinical staging and management of Hodgkin's disease and lymphosarcoma. *New Engl. J. Med.* **271,** 327 (1964).
4. K. Musshoff, H. Schmidt-Vollmer and D. Merten, Reticulum cell sarcoma, an oncologic model for a system of classifying the malignant lymphomas. *Europ. J. Cancer* **7,** 451 (1971).
5. B. Modan, M. Shani, B. Goldman and M. Modan, Nodal and extranodal malignant lymphomas in Israel: an epidemiological study. *Brit. J. Haemat.* **16,** 53 (1969).

6. M. V. PETERS, R. HASSELBACK and T. C. BROWN, The natural history of the lymphomas related to the clinical classification. In *Proceedings of the International Conference on Leukemia-Lymphoma* (Edited by C. J. D. ZARAFONETIS), pp. 357–370, Lea & Fibiger, Philadelphia, 1968.
7. S. A. ROSENBERG, H. D. DIAMOND and L. F. CRAVER, Lymphosarcoma: The effects of therapy and survival in 1,269 patients in a review of 30 years experience. *Ann. int. Med.* **53,** 877 (1960).
8. S. A. ROSENBERG, H. D. DIAMOND, B. JASLOWITZ and L. F. CRAVER, Lymphosarcoma: A review of 1269 cases. *Medicine* **40,** 31 (1961).
9. H. SHOJI and T. R. MILLER, Primary reticulum cell sarcoma of bone. *Cancer (Philad.)* **28,** 1234 (1971).
10. B. V. R. WERF-MESSING, Reticulum cell sarcoma and lymphosarcoma. A retrospective study of potential survival in loco-regional disease. *Europ. J. Cancer* **4,** 549 (1968).
11. A. LIPTON and B. J. LEE, Prognosis of stage I lymphocarcoma and reticulum cell sarcoma. *New Engl. J. Med.* **284,** 230 (1971).
12. J. W. BERG, D. SCHOTTENFELD, R. V.P. HUTTER and F. W. FOOTE, Histology, epidemiology, and end results. The Memorial Hospital Cancer Registry, N.Y. Memorial Hospital for Cancer and Allied Disease, 1969.
13. G. FREMAN, J. W. BERG and S. J. CUTTER, Occurrence and prognosis of extranodal lymphomas. *Cancer (Philad.)* **29,** 252 (1972).
14. G. E. HANKS, L. N. TERRY, J. A. BRYAN and J. F. NEWSOME, Contribution of diagnostic laparotomy to staging non-Hodgkins lymphoma. *Cancer (Philad.)* **29,** 41 (1972).
15. S. A. ROSENBERG, Report of the committee on the staging of Hodgkin's disease. *Cancer Res.* **26,** 1310 (1966).
16. P. P. CARBONE, H. S. KAPLAN, K. MUSSHOFF, D. W. SMITHERS and M. TUBIANA. Report of the committee on Hodgkin's disease classification. *Cancer Res.* **31,** 1860 (1971).

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Leucocyte Ascorbic Acid and Urinary Hydroxyproline Levels in Patients Bearing Breast Cancer with Skeletal Metastases*

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Abstract—*The leucocyte ascorbic acid and urinary hydroxyproline were determined in a group of patients bearing breast tumour with skeletal metastases. These results have been compared with those of the patients bearing advanced malignant tumours at other sites, the healthy subjects and the hospital patients with nonmalignant diseases. The leucocyte ascorbic acid levels were found to be very low and the urinary hydroxyproline levels very high in the patients bearing breast tumours. The increased levels of hydroxyproline in the urine of these patients were reversed by a loading dose of ascorbic acid.*

INTRODUCTION

THERE IS some direct or indirect evidence to suggest that ascorbic acid may be important in stress [1]. Although there have been a number of reports on the assessment of the vitamin nutriture in various conditions, few or perhaps no studies have been concerned with the patients bearing malignant diseases. The present study was conducted to investigate the ascorbic acid nutrition in a group of cancer patients with particular reference to those bearing breast tumours with skeletal metastases. As the leucocyte ascorbic acid (LAA) is generally regarded as the most reliable index of the vitamin nutrition [2], this has been measured in the patients investigated. Ascorbic acid has been reported to be involved in the synthesis of collagen [3, 4] which is the major extracellular constituent of bone matrix. Since collagen contains a high level of hydroxyproline, the present investigation has been extended to study whether there is any correlation between the LAA level and the level of hydroxyproline in urine (UHP), and whether this correlationship can indicate the presence of skeletal metastases in the patients with breast tumour.

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PATIENTS AND METHODS

A group of 22 advanced breast cancer patients with radiologically demonstrable skeletal metastases and a group of 26 patients bearing milgnant tumours in other sites, such as brain, lung, throat, stomach, rectum and cervix without any skeletal metastasis, were studied. All patients had at some time received appropriate surgical, radiational or drug treatment. As far as could be determined, all the patients had taken a similar diet; the patients with breast tumours had also been receiving vitamin supplements. Heparinized blood samples and fresh overnight specimens of urine were collected from these patients. Similarly, urine and blood specimens were collected from 10 healthy subjects and 10 hospital patients without cancer but with debilitating diseases, such as tuberculosis, chronic bronchitis and coronary thrombosis.

Estimations of ascorbic acid and total hydroxyproline

The leucocyte ascorbic acid levels were estimated by the method of Denson and Bowers [5]. The ascorbic acid in urine was measured by the procedure based on the coupling reaction of ascorbic acid with 2:4-dinitrophenylhydrazine to form a chromagenic compound [6]. Total hydroxyproline was measured in urine following the method described by Stegemann and Salder [7]. The method involves the oxidation of hydroxyproline to pyrrole compounds which can then be used to form a specific colour reaction with *p*-dimethylaminobenzaldehyde.

As it was not possible to collect a 24 hr urine specimen from each subject, all results in urine specimens were expressed/*g* creatinine, which is normally accepted as being extremely constant in its excretion.

Effect of a loading dose of ascorbic acid on the urinary hydroxyproline levels

A dose of one gram ascorbic acid was administered by mouth to each patient. For administrative convenience this experiment was started at 10 a.m. in order to ensure that the morning blood and urine samples could be taken before the vitamin was administered orally. Urine was collected at four-hourly intervals for 12 hr after the administration of ascorbic acid. The hydroxyproline level was measured in all urine specimens.

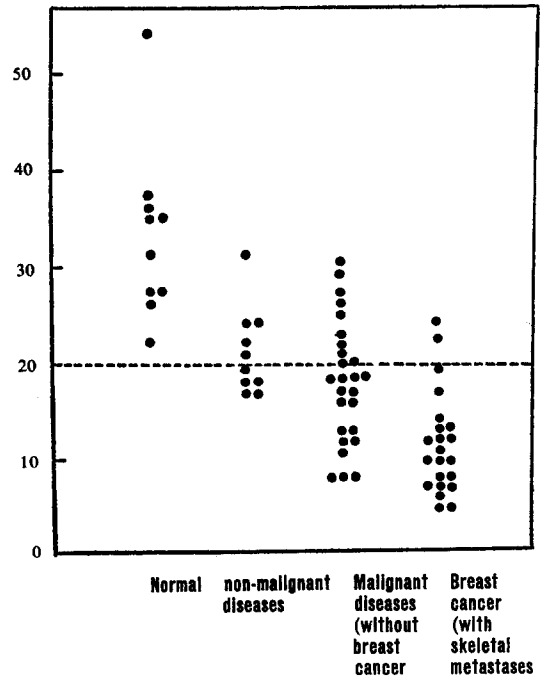


Fig. 1. *Leucocyte ascorbic acid levels in advanced cancer patients.*

Table 1. *Particulars of the patients studied*

Cases	Osseous deposits	No. of cases	Sex	Age (year)
Normal	—ve	10	7M + 3F	58 (48–68)
Non-malignant	—ve	10	6M + 4F	64 (52–78)
Malignant (without breast cancer)	—ve	26	15M + 11F	62 (55–72)
Breast cancer	+ve	22	22F	66 (55–85)

All subjects except the normal ones were in hospital during the investigations. As these patients had standard hospital diets, it was assumed that their ascorbic acid intake from the dietary sources were roughly similar.

RESULTS

The results of the LAA levels of the various groups of patients investigated (Table 1) are depicted graphically in Fig. 1. Of the groups investigated, the highest levels of LAA were observed in the healthy subjects (average 33 $\mu g/10^8$ W.B.C.) and lowest in the patients bearing breast cancer with skeletal metastases (average 12 $\mu g/10^8$ W.B.C.). In the patients with nonmalignant diseases and with malignant diseases other than breast cancer, the LAA levels were found to be in between the two groups (average 21 and 17 $\mu g/10^8$ W.B.C., respectively).

The total UHP levels, however, followed an opposite pattern to the LAA levels (Fig. 2). Thus the lowest levels of UHP were observed in the healthy subjects (average 16 mg/g creatinine) and the highest were observed in the patients with breast tumour accompanied by bone metastases (135 mg/g creatinine). The average UHP levels of the patients with other malignant diseases were similar to those of the patients with non-malignant diseases (46 mg/g creatinine).

Table 2 shows the effect of a loading dose of ascorbic acid on the UHP levels of five breast tumour patients who were deficient in LAA (below 20 $\mu g/10^8$ W.B.C.) and accompanied by bone metastases, and five patients bearing malignant tumours in other sites without skeletal metastases who had normal LAA levels (above 20 $\mu g/10^8$ W.B.C.). One gram of ascorbic acid was administered to each of these patients by mouth. The UHP levels of the patients bearing breast tumour with secondary bone metastases were markedly decreased 4 hr after the vitamin was taken. The vitamin administration did not, however, result in any significant change in the UHP levels of the

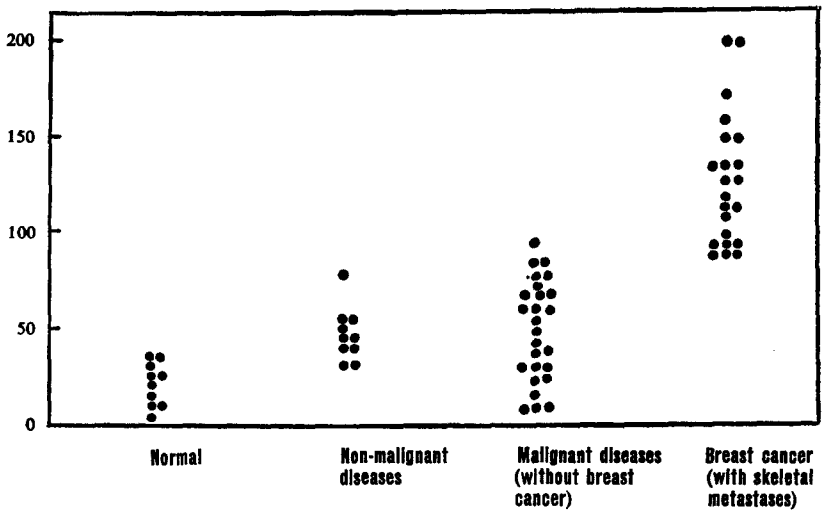


Fig. 2. Urinary excretory levels of total hydroxyproline in advanced cancer patients.

patients bearing tumours at other sites without skeletal metastases.

Table 2. Urinary excretory levels of hydroxyproline after a loading dose of ascorbic acid

Case no.	Site of tumour	LAA level (μg/10^8 W.B.C.)	UHP level (hours after a dose of ascorbate) 0	4	8	12
1.	Breast	9·8	84	50	46	52
2.	Breast	6·0	160	72	54	49
3.	Breast	5·8	260	65	13	22
4.	Breast	7·2	112	78	56	42
5.	Breast	10·3	91	47	43	33
6.	Larynx	30·2	35	32	32	36
7.	Pancreas	22·0	50	46	64	57
8.	Bronchus	27·3	38	35	30	30
9.	Rectum	20·2	40	32	30	30
10.	Rectum	23·2	20	12	12	18

One gram of ascorbic acid was administered to each patient. Urine was collected at four-hourly intervals for 12 hr after the vitamin intake. Urinary excretory levels of hydroxyproline were expressed as mg/g. creatinine.

DISCUSSION

The present studies have demonstrated that the UHP levels are strikingly elevated in patients with breast cancer who have radiologically demonstrable osseous deposits. Similar observations have also been made by others [8, 9, 10]. The skeletal disease is known to cause bone resorption, and this involves extensive loss of bone matrix [11]. It is therefore possible that the increased level of UHP in the patients bearing breast tumour with secondary bone metastases may be due to increased rate of degradation of bone collagen rather than the breast tumour itself; this hypothesis may be supported by the fact that collagen breakdown in disorders affecting bone such as hyperthyroidism, Paget's disease, osteomalacia, rickets and Fanconi's syndrome has also been reported to be associated with increased urinary excretion of hydroxyproline [12, 13, 14]

It is noteworthy that the LAA levels are low while the UHP levels are markedly high in the patients with skeletal metastases. The significance of these results is not, however, clear. Nevertheless, it has been suggested that ascorbic acid is required to protect collagen molecule from breakdown [15]. If this is so, then it is not surprising to observe that the patients with skeletal metastases, deficient in ascorbic acid according to their LAA levels, had high urinary levels of hydroxyproline which is a common breakdown product of collagen. However, the way in which ascorbic acid protects the collagen molecule from its breakdown, is not known at the present time. Nevertheless, it is probable that the turnover rate of collagen synthesis may be a rate limiting factor for collagen being broken down by bone metastases, and ascorbic acid is involved in maintaining the rate of collagen synthesis by hydroxylation of proline. This hypothesis is supported by the observation in the present study that the administration of a loading dose of ascorbic acid to the patients with skeletal metastases and subnormal LAA levels resulted in a striking drop in UHP to normal levels. However, the UHP levels of the patients without bone metastases, who had normal levels of LAA to begin with, remained unaffected after a dose of the vitamin. These results may be due to the fact that the patients without bone metastases may have been

refractory to effects of excess ascorbic acid, whereas the patients with bone metastases were all clearly vitamin deficient.

The results of the UHP levels in the breast cancer patients with skeletal metastases are, however, somewhat different from those of other conditions with no clinical bone diseases. Thus, Windsor and Williams [16] reported that UHP levels were low in the elderly with low LAA. Furthermore, scorbutic guinea pigs also have been found to be accompanied by low UHP levels [17]. This would suggest that bone metastasis patients have an elevated UHP inspite of the low LAA rather than because of it.

Whatever the final explanation of the results may be, two obvious questions emerge from the present investigations. These are: whether large doses of ascorbic acid can retard the rate of skeletal metastases; and whether the patients bearing breast tumour may have increased requirements for the vitamin.

Recently, in a course of a clinical trial, Cameron and Baird [18] observed that a group of patients with multiple skeletal metastases, were relieved from pain when administered 10 grams of ascorbic acid daily for about a week. These workers also claimed that regular narcotic analgesics treatment to the patients could be discontinued without inducing withdrawal symptoms. These clinical observations, in addition to our demonstrations that the increased levels of hydroxyproline in urine of the patients bearing breast tumours with skeletal metastases can be reversed by a loading dose of ascorbic acid, may lead to a suggestion that large doses of the vitamin may retard the rate of skeletal metastases.

All the patients bearing breast tumour included in the present investigation had received vitamin supplements. Despite the fact that these patients were receiving extra vitamins in their diets, the LAA levels were very low. It is therefore probable that the patients may have increased requirements for ascorbic acid, perhaps to maintain the turnover rate of synthesis of bone collagen which may be continually being broken down by the invasion of tumour.

Undoubtedly, that our results are sufficiently interesting to merit further investigation and may prove to have practical implications for the treatment of patients with bone metastases.

REFERENCES

1. E. M. Baker, Vitamin C requirements in stress. *Amer. J. clin. Nutr.* **20,** 583 (1967).
2. W. Bartley, H. A. Krebs and J. R. P. O'Brien, Vitamin C requirement of human adults. M.R.C. Special series. No. 28 (1953).
3. B. S. Gould and J. F. Woessner, Biosynthesis of collagen: The influence of ascorbic acid on the proline, hydroxyproline, glycine and collagen content of regenerating guinea pig skin. *J. biol. Chem.* **226,** 289 (1957).
4. W. V. B. Robertson, J. Hewitt and C. Herman, Relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carrageaman granuloma. *J. biol. Chem.* **234,** 105 (1959).
5. K. W. Denson and E. F. Bowers, The determination of ascorbic acid in white blood cells: a comparison of W.B.C. ascorbic acid and phenolic acid excretion in elderly patients. *Clin. Sci.* **21,** 157 (1961).
6. J. H. Roe and C. A. Kuether, The determination of ascorbic acid in whole blood and urine through the 2,4–dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. biol Chem.* **147,** 399 (1943).
7. H. Stegemann and K. Stalder, Determination of hydroxyproline. *Clin. chim. Acta.* **18,** 267 (1967).
8. G. Bonadonna, M. J. Merlino, W. P. Laird Myers and M. Sonenberg, Urinary hydroxyproline and calcium metabolism in patients with cancer. *New. Engl. J. Med.* **275,** 298 (1966).
9. H. F. Hosley, E. G. Taft, K. B. Olson, S. Gates and R. T. Beebe, Hydroxyproline excretion in malignant neoplastic disease. *Arch. intern. Med.* **118,** 565 (1966).
10. C. Cuschieri, Urinary hydroxyproline excretion in early and advanced breast cancer—A sequential study. *Brit. J. Surg.* **60,** 800 (1973).
11. J. F. Woessner, Biological mechanisms of collagen resorption, In *Treatise on Collagen: Biology of Collagen* (Edited by B. S. Gould), Academic press, London, New York) p. 253 (1968).
12. T. A. Dull and P. H. Henneman, Urinary hydroxyproline as an index of collagen turnover in bone. *New Engl. J. Med.* **268,** 132 (1963).

13. L. KLEIN, General correlation of serum alkaline phosphatase and urinary hydroxyproline in metabolic bone diseases, *J. clin. Invest.* **43,** 1250 (1964).
14. P. CRABBE and K. J. ISSELBACHER, Urinary hydroxyproline excretion in malabsorption states, *Gastroenterology* **48,** 307 (1965).
15. J. GROSS, Formation of collagen: IV. Effect of vitamin C deficiency on the natural salt-extractable collagen of skin. *J. exp. Med.* **109,** 557 (1959).
16. A. C. W. WINDSOR and C. B. WILLIAMS, Urinary hydroxyproline in the elderly with low leucocyte ascorbic acid levels. *Brit. Med. J.* **1,** 732 (1970).
17. G. R. MARTIN, S. E. MERGENHAGEN and D. J. PROCKOP, Influence of scurvy and Lathyrism (Odoratism) on hydroxyproline excretion. *Nature, (Lond.)* **191,** 1008 (1961).
18. E. CAMERON and G. M. BAIRD, Ascorbic acid and dependence on opiates in patients with advanced disseminated cancer. *J. internat. Res. Commun. August,* 38 (1973).

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A New Pharmacological Method of Host Conditioning in order to Facilitate *In Vivo* Growth of Fibroblast-like Cells Cultivated *In Vitro*

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Abstract—*A short intensive treatment with N-diazoacetylglycine amide (DGA) before the inoculum, followed by prolonged daily administration of* L-*asparaginase (Asnase), was tested for its ability in allowing the demonstration of tumorigenic properties of fibroblast-like cells cultured* in vitro. *With such an immunodepressive treatment, progressive tumor growth was obtained in syngeneic mice injected with cells of two spontaneously transformed sublines apparently devoid of tumorigenic potential in untreated mice.*

INTRODUCTION

NEOPLASTIC transformation of normal cells in tissue culture by a variety of conditions or agents is correlated to the appearance of new characteristics by the transformed cells. Among these are: (1) stable and unlimited growth *in vitro* [1]; (2) lack of contact inhibition between cells [2]; (3) increased cloning efficiency in fluid medium and soft agar [3]; (4) ability to multiply at 41°C [4]; (5) loss of ability to form colonies on glutaraldehyde-fixed normal cells [5]; (6) agglutinability by concanavalin A [6]; (7) multilayerd and crisscrossed arrangement of cells [7]; and (8) changes in cell morfology [8]. Although some of these alterations have proved useful in the monitoring of transitional stages, their relation to carcinogenesis remains undefined. Therefore, the only conclusive assay of neoplastic transformation of cells *in vitro* is to determine their capacity to grow progressively as invasive neoplasms on implantation into compatible hosts. However, neoplastic transformation is usually accompanied by the synthesis of tumor transplantation antigens which can cause rejection of the cells implanted in syngeneic recipients. To circumvent the immune response elicited by such new antigenicities, the inoculation of cells is generally performed into newborn animals or into X-ray and/or cortisone-treated animals.

This work was undertaken to evaluate the ability of *N*-diazoacetylglycine amide (DGA) in combination with L-asparaginase (Asnase) in allowing the growth of fibroblast-like transformed cells in syngeneic mice. The pharmacological properties of the two agents seem particularly fit for this aim. DGA exerts a marked and long lasting immunodepressive activity when administered before the antigenic stimulus [9, 10], and since its half life is approximately 15 minutes, (unpublished data), any interference of the drug with the implanted cells is excluded. Asnase that is devoid of carcinogenetic activity in mice [11], has a narrow spectrum of citotoxicity (essentially on lymphoid cells), and significantly depress the immune response elicited by transplantation antigens when administered, at relatively low doses, after the antigen for prolonged periods [12, 13]. Because of the different systemic effects of the two compounds, their combined use is not likely to result in additive toxicity.

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MATERIAL AND METHODS

A fibroblast-like cell line, (B6–1), established after 30 subcultures of a primary C57BL/6 Ar embryo culture, was used to derive the following sublines: B6–4, transformed by treatment with methylcholanthrene, and B6–20 and B6–21, two sublines which spontaneously transformed and were cloned twice. Cells were grown in Eagle's minimum essential medium with 20% calf serum without antibiotics, in prescription bottles incubated at 37°C. The tumorogenicity of the cultured cells was tested by their subcutaneous inoculation into the right flank of C57BL/6 Ar inbred mice, aged 3–4 months. Cell suspensions with more than 10% of cells stained by trypan bleu were discarded. DGA and Asnase (Crasnitin Bayer) were dissolved in isotonic saline immediately before use in a concentration that could be administered on the basis of 0·02 ml/g of body weight, and were injected i.p. Mice were given 500 mg/kg per day of DGA throughout the four days before the inoculum and 3000 I.U./kg per day of Asnase for periods variable from 20 to 40 consecutive days, starting from the day of cell injection. Subcutaneous tumours were inspected weekly and their volumes calculated in mm^3 according to the formula

$$[(d_1 - 0{\cdot}5)\ (d_2 - 0{\cdot}5)]^{3/2}\ \pi/6$$

where d_1 and d_2 are the longest and the shortest axes in mm and 0·5 is a correction factor for the double thickness of the skin.

RESULTS

The results of the animal assays are summarized in the table. MCA-transformed B6–4 cells produced progressive tumor growth in 13 out of 14 syngeneic normal mice injected with $1–1{\cdot}4 \times 10^5$ cells and in 4 out of 5 mice injected with 10^4 cells. On the contrary, progressive tumor growth did not take place in 19 out of

Table 1. *Tumor production by subcutaneous injection of cells of the sublines B6–4, B6–20, and B6–21 into normal and DGA + ASNASE–immunodepressed syngeneic adult C57BL/6Ar mice*

Subline (*a*)	Inoculum size (cells $\times 10^{-5}$)	No. of mice and sex	Treatment (*b*)	Average tumor volume (range) in mm^3 at day: +13	+25	+50	+75	No. of mice without tumor at day 100
				No. of mice with tumor				
B6-4(10)	0·1	5♂	None	—	9(—)/1	362(8–902)/4	558(70–1012)/4	1
	1	5♂	None	—	100(4–158)/5	1054(47–1360)/5	2126(144–3299)/5	0
	1·4	9♀	None	8(0·2–26)/9	101(50–163)/9	1843(44–3256)/9	5155(64–5465)/7(*c*)	0
B6-20(9)	40	5♂	DGA + Asn.(40)	118(57–263)/5	561(231–730)/5	3997(2066–5525)/4(*c*)	7392(5337–10169)/4	0
		3♂	None	176(57–300)/3	60(—)/1	—	—	3
B6-21(6)	0·1	6♂	DGA + Asn.(40)	—	0,5(—)/1	262(44–475)/4	1254(124–2178)/4	2
		6♂	None	—	—	—	—	6
B6-21(7)	5	6♀	DGA + Asn.(30)	10(4–20)/6	32(18–47)/6	552(34–2618)/6	725(176–1403)/5(*c*)	0
		6♀	None	3(0,8–8,4)/5	—	—	—	6
B6-21(8)	20	5♂	DGA + Asn.(20)	50(27–103)/5	85(37–208)/5	747(178–1992)/4(*c*)	2722(883–5058)/4	0
		5♀	None	—	—	—	10(—)/1	4

(*a*) The number in parentheses is indicative of the number of sucultures performed.

(*b*) Mice were given 500 mg/Kg/day of DGA on the 4 days before the inoculum, and 3000 IU/Kg/day of ASNASE for the number of days indicated in parentheses, starting from the day of cell inoculation.

(*c*) The missing animals were killed for histological examination.

20 normal syngeneic mice transplanted with various numbers of cells of the two spontaneously transformed sublines B6–20 and B6–21, except for the appearance of nodules, which later regressed, in mice implanted with 40×10^5 B6–20 cells. Among untreated mice inoculated with 20×10^5 B6–21 (8) cells, at the 75th day, a single animal showed a little nodule that persisted unmodified until the end of the observation period.

The immunodepression of the recipent with DGA and Asnase allowed a clear demonstration of the tumorigenic potential of the two spontaneously transformed sublines. Progressive tumor growth took place in 4 of 6 mice injected with 10^4 B6–21 cells and in all the 16 implanted with higher numbers of cells. Out of the 22 mice treated with DGA and Asnase, as indicated in the table, no death was observed and the body weight losses ranged from 10 to 15% at two weeks after the inoculum.

The present experiments have stressed the need of an effective immunodepression of the recipients in order to reveal neoplastic transformations of low tumorigenic potential, and have indicated that a treatment with DGA and Asnase can be considered a suitable conditioning procedure to reach this goal.

REFERENCES

1. L. Hayflick and P. S. Moorhead, The serial cultivation of human diploid cell strains. *Expl. Cell Res.* **25,** 585 (1961).
2. S. A. Aaronson and G. J. Todaro, Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. *Science* **162,** 1024 (1968).
3. Z. Rabinowitz and L. Sachs, The formation of variants with a reversion of properties of transformed cells. V. Reversion to a limited life-span *Int. J. Cancer* **6,** 388 (1970)
4. L. Ossowski and L. Sachs, Temperature sensitivity of polyoma virus, induction of cellular DNA synthesis, and multiplication of transformed cells at high temperature. *Proc. nat. Acad. Sci. U.S.* **58,** 1938 (1967)
5. Z. Rabinowitz and L. Sachs, Reversion of properties in cells transformed by polyoma. *Nature (Lond.)* **220,** 1203 (1968).
6. M. Inbar and L. Sachs, Interaction of the carbohydrate-binding protein Concanavalin A with normal and transformed cells. *Proc. nat. Acad. Sci. U.S.* **63,** 1418 (1969).
7. T. T. Chen and C. Heidelberger, *In vitro* malignant transformation of cells derived from mouse prostate in the presence of 3-methylcholanthrene. *J. nat. Cancer Inst.* **42,** 915 (1969).
8. B. E. Barker and K. K. Sanford, Cytologic manifestation of neoplastic transformation *in vitro*. *J. nat. Cancer Inst.* **44,** 39 (1970).
9. G. Brambilla, S. Parodi, M. Cavanna and L. Baldini, The immunodepressive activity of *N*-diazoacetylglycine amide in some transplantation systems. *Transplantation* **10,** 100 (1970).
10. L. Baldini, G. Brambilla, M. Cavanna, C. E. Caraceni and S. Parodi, Effect of *N*-diazoacetylglycine amide on the immune response of mice to sheep red blood cells. *Transplantation* **13,** 224 (1972).
11. G. Brambilla, C. E. Caraceni and S. Parodi, Valutazione nel topo Swiss neonato dell'attivita carcinogena di alcuni composti antineoplastici ed immunodepressivi. *Boll. Soc. ital. Biol. sper.* **47,** 418 (1971).
12. E. M. Hersh, Immunosuppression by L-asparaginase and related enzymes. *Transplantation* **12,** 368 (1971).
13. G. Brambilla, S. Parodi, M. Cavanna, C. E. Caraceni and L. Baldini, The immunodepressive activity of *Escherichia coli* L-asparaginase in some transplantation systems. *Cancer Res.* **30,** 2665 (1970).

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Redistribution of Immunoglobulin Determinants on Human Lymphocytes in Lymphoproliferative Disorders*

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Abstract—*Using ^{125}I- or FITC-labelled anti-immunoglobulin antisera we studied the redistribution of surface Ig–anti-Ig complexes on the surface of human normal and neoplastic B lymphocytes, obtained from patients with chronic lymphocytic leukaemia and lymphosarkoma. The results were compared with those obtained from murine spleen cells. Whereas the majority of mouse lymphocytes exhibited cap formation under appropriate conditions, this staining pattern was only demonstrable on a very small, but distinct percentage of normal human lymphocytes and lymphosarcoma cells. In contrast typical cap formation was not observed on leukaemic blood lymphocytes. These findings are discussed with respect to the functional impairment of leukaemic lymphocytes.*

INTRODUCTION

ON MURINE B-cells the redistribution of surface immunoglobulin (Ig) molecules, after reaction with labelled antibodies, has been studied extensively by several groups [1–6].

Under appropriate conditions crosslinking of surface molecules by bivalent antibodies resulted in "cap formation" at distinct areas overlying the Golgi field. On human peripheral blood lymphocytes a different spacing of Ig-determinants on the cell surface has been reported [7], which makes these cells less capable of cap formation. Alterations of the membrane structure of lymphocytes in chronic leukaemia (CLL) were postulated by various investigators [8, 9, 10, 11, 12], but the fate of Ig-determinants after reaction with antibodies has not yet been reported in detail. We studied the redistribution of Ig- anti Ig complexes on the surface of lymphocytes of patients with lymphoproliferative disorders. Results were compared with those obtained from normal human and mouse lymphocytes.

METHODS

Column separated normal blood lymphocytes, blood cells from patients with chronic lymphocytic leukaemia (CLL) and lymph node suspensions from patients with lymphosarcoma, showing surface Ig of the μk-type on the majority of cells, as well as murine spleen cells were tested. 2×10^6 lymphocytes and labelled anti-Ig antibody, resuspended in a total volume of 100 μl tissue culture medium TC 199, were incubated at 4°C for 30 min. At the same time, cells were incubated with the same amount of unlabelled rabbit antibody; they were washed and then stained in the cold with labelled anti-rabbit Ig antibody in excess. A rabbit anti- mouse k-chain antiserum (RAM-K, 13,), ^{125}I- labelled anti- human k- or μ-chain antisera (RAH-k, RAH-μ, [14]) and a ^{125}I- or FITC- labelled swine anti-rabbit Ig antiserum (SARIg, Fa. Dakopats, Denmark) were used. For optimal capping conditions various amounts of antibodies including con-

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centrations with marked antibody excess ([14], see also Fig. 1) were employed. After 4 washings in the cold cells were resuspended in 2·0 ml TC 199 supplemented with 10% fetal calf serum and incubated at 37°C. After various intervals of incubation, cells were harvested and examined for surface Ig distribution by autoradiography [14] or immunofluorescence

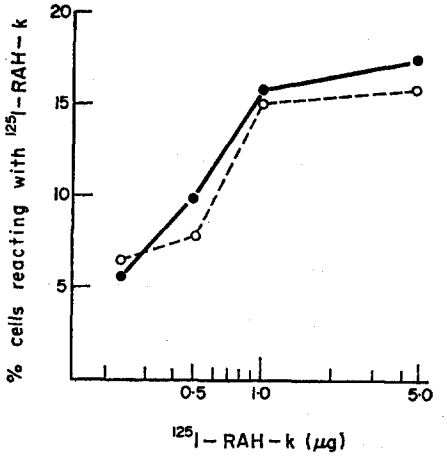


Fig. 1. Correlation between the dose of ^{125}I–RAH-k antibody and the percentage of labelled normal human blood lymphocytes. Data of two controls are given. With this antiserum the mean percentage of stained normal blood lymphocytes was 18% [14].

[13]. Cap formation was considered, if staining was confined to less than 1/4 of the cell surface [5]. Cap formation was a rare event on human lymphocytes. Intermediate stages between caps and diffuse staining, however, were often observed, but only under conditions favouring cap formation and were classified as follows (see also Fig. 2): less than 1/4 of the cell surface stained—typical caps 1/4–1/2 of the cell surface stained—incomplete caps more than 1/2 of the cell surface stained, with preferential staining over one pole—intermediate forms—diffuse staining.

RESULTS

Treatment of a constant number of blood lymphocytes with increasing amounts of the labelled antibody resulted in an increase of staining intensity and percentage of labelled cells, until a saturation point was reached (Fig. 1). Higher doses of antibody did not further increase the percentages of stained cells. Crosslinking of the majority of surface Ig molecules appeared necessary for optimal cap formation. Amounts of antibody 2–4 times those required for obtaining saturation doses

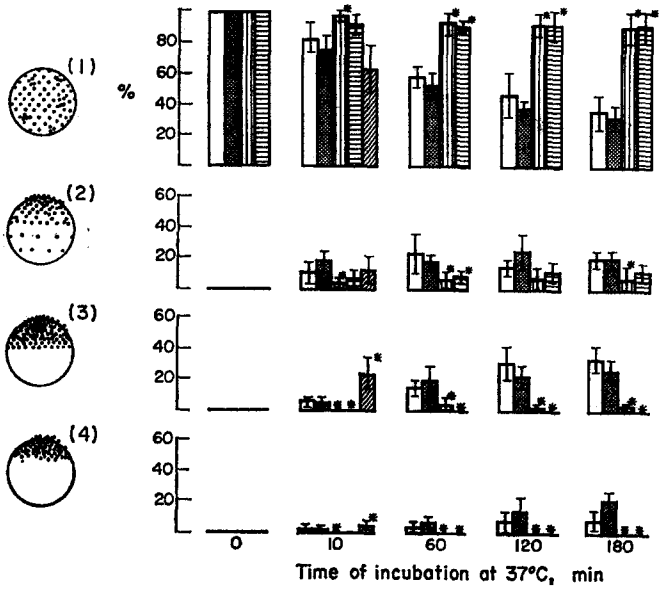


Fig. 2. Redistribution of Ig-determinants on human normal and neoplastic lymphocytes
□ ⬚ *normal blood lymphocytes*
▮ ▤ *leukaemic blood lymphocytes*
▨ *lymphosarcoma cells, obtained from lymph node biopsies*
□ ▮ ▨ *direct staining technique*
⬚ ▤ *sandwich technique*
1. *"diffusely stained cells"*
2. *"intermediate forms"*
3. *"incomplete caps"*
4. *"typical caps" (for details of definition see under methods)*
5. *P values versus normal controls* > 0·05.

Table 1. *Redistribution of Ig-determinants: comparison of normal human and mouse lymphocytes with cells obtained from patients with chronic lymphocytic leukaemia and lymphosarcomas*

Cell source	Antisera	Experiments No.	Percentage of polar labelled cells* (mean values) Time of incubation at 37°C (min) 0	10	60	120	180
Mouse spleen cells	RAM-k+FITC-SARIg	5	<1(-)	88(12)	—†	—	—
Human normal blood	^{125}I-RAH-μ	5	0(<1)	1(16)	—	—	—
lymphocytes	^{125}I-RAH-k	6	0(<1)	2(15)	3(40)	7(51)	8(62)
	RAH-k+^{125}I-SARIg	6	0(<1)	2(23)	6(43)	16(59)	21(67)
Human leukaemic blood	^{125}I-RAH-μ	8	0(<1)	0(3)	—	—	—
lymphocytes	^{125}I-RAH-k	5	0(<1)	0(3)	0(7)	0(5)	0(8)
	RAH-k+^{125}I-SARIg	5	0(<1)	0(8)	0(7)	0(9)	0(10)
Human lymphosarcoma	^{125}I-RAH-μ	5	—	5(25)	—	—	—
lymph node cells	^{125}I-RAH-k	5	—	4(31)	—	—	—

*Numbers of typical cap forming cells and in brackets percentages of cells with "incomplete caps" (for definition see methods) are given.
†Not done.

were therefore employed during our subsequent studies.

At 4°C normal and pathological human, as well as murine lymphoid cells, showed a homogenous distribution of label. After a short incubation at 37°C the majority of mouse spleen cells showed typical cap formation. After 10 min of incubation approximately 90% of murine B-cells formed caps (Table 1). Polar distribution of the label defined as caps was rarely seen on human normal blood lymphocytes after this short time of incubation (Table 1 and Fig. 2). A considerable higher percentage of human blood lymphocytes (between 12 and 16%) showed a partial redistribution of label ("incomplete cap formation"). During prolonged incubation intervals at 37°C an increasing percentage of cells was observed with this staining pattern and labelling intensity decreased. Nevertheless, the marked differences in the redistribution of the label between murine and human lymphocytes remained even after an incubation time of 180 min.

Staining of leukaemic lymphocytes with anti-Ig antisera and the redistribution of label differed from normal human blood lymphocytes. The density of both μ- and k-determinants was markedly decreased in 4 out of 8 CLL patients. Polar redistribution of the stain was a very rare finding even after long incubation, and labelling intensity decreased more slowly. Typical caps were not observed and the percentages of leukaemic lymphocytes with "incomplete caps" were considerably lower in comparison to normal blood lymphocytes.

The formation of uropods, which carried usually the whole label, was frequent on murine spleen cells; they were occasionally seen on normal human lymphocytes and not observed on leukaemic cells. In contrast, lymphosarkoma cells obtained from biopsies showed a normal or increased staining after incubation with anti-Ig antisera [14]. The percentage of lymphosarcoma cells with polar staining pattern was comparable or somewhat higher in comparison to normal blood lymphocytes.

DISCUSSION

Cap formation depends on various factors including density and spatial distribution of surface molecules [1–7]. The redistribution of Ig-anti Ig complexes after incubation of normal human lymphocytes was strikingly less pronounced in comparison to murine lymphocytes. This may be caused by the different distribution of surface immunoglobulins in mice and man [7]. Nevertheless, on normal human lymphocytes a small, but distinct percentage of cells showed a polar distribution of label, which increased with prolonged incubation. Beside the process of cap formation various cellular functions as endocytosis with subsequent enrichment of Ig-anti Ig complexes at distinct intracellular areas, as well as partial shedding of the label may cause a polar staining pattern. Whereas endocytosis and/or shedding of label are presumably not responsible for the redistribution observed within a few minutes, these processes may influence the staining pattern after longer incubation periods.

On leukaemic lymphocytes even a partial redistribution of the stain was rarely observed. The density of surface Ig molecules was reduced in some CLL patients [17, 14]. However, in patients with lymphocytes exhibiting high concentrations of anti-Ig binding sites the

redistribution of label was markedly impaired. Moreover, a wide range of antibody concentrations as well as sandwich techniques were employed for facilitating lattice formation, but the redistribution of the label was not improved. B-lymphocytes in our patients with lymphosarcomas exhibited membrane pattens comparable to normal controls. Marked differences in staining density and membrane redistribution of lymphosarcoma cells and CLL lymphocytes were observed.

The significance of cap formation has not yet been established in detail. Its role in the early steps of lymphocyte transformation has been considered, but yet not conclusively demonstrated [4, 5, 15]. Membrane fluidity as assessed by the capacity for cap formation may be related to cell motility [4, 5, 6, 16]. An impaired recirculation of leukaemic lymphocytes has been reported [17], and in this respect our finding of an impaired redistribution of Ig-determinants deserves particular interest.

We hope that further studies on membrane dynamics and structure will enable a better understanding of the functional impairment of leukaemic cells.

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REFERENCES

1. R. B. Taylor, P. H. Duffus, M. C. Raff and S. de Petris, Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature New Biol.* **233,** 225 (1971).
2. E. R. Unanue, W. D. Perkin and M. J. Karnovsky, Ligand induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. exp. Med.* **136,** 885 (1972).
3. M. J. Karnovsky, E. R. Unanue and M. Leventhal, Ligand induced movement of lymphocyte membrane macromolecules. II. Mapping of surface moieties. *J. exp. Med.* **136,** 907 (1972).
4. F. Loor, L. Forni and B. Pernis, The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Europ. J. Immunol.* **2,** 203 (1972).
5. S. de Petris and M. C. Raff, Distribution of immunoglobulin on the surface of mouse lymphoid cells as determined by immunoferritin electron microscopy. Antibody-induced, temperature dependent redistribution and its implications for membrane structure. *Europ. J. Immunol.* **2,** 523 (1972).
6. E. R. Unanue, M. J. Karnovsky and H. D. Engers, Ligand induced movement of lymphocyte membrane macromolecules. III. Relationship between the formation and fate of anti-Ig surface Ig complexes and cell metabolism. *J. exp. Med.* **137,** 675 (1973).
7. K. A. Aulth, M. J. Karnovsky and E. R. Unanue, Studies on the distribution of surface immunoglobulins on human B-lymphocytes. *J. clin. Invest.* **52,** 2507 (1973).
8. G. Michlmayr and H. Huber, Receptor sites for complement on certain human peripheral blood lymphocytes. *J. Immunol.* **105,** 670 (1970).
9. A. C. Aisenberg and K. J. Bloch, Immunoglobulins on the surface of neoplastic lymphocytes. *New Engl. J. Med.* **287,** 272 (1972).
10. G. D. Ross, E. M. Rabellino, M. J. Polley and H. M. Grey, Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocyte rosette-forming cells in normal and leukaemic human lymphocytes. *J. clin. Invest.* **52,** 377 (1973).
11. M. Schweitzer, C. J. M. Melief and V. P. Eijsvogel, The nature of the transforming lymphocyte in chronic lymphocytic leukaemia. *Europ. J. Immunol.* **3,** 121 (1973).
12. H. D. Menne and H. D. Flad, Membrane dynamic of HL-A anti H1L-A complexes of human normal and leukaemic lymphocytes. *Clin. exp. Immunol.* **14,** 57 (1973).
13. Ch. Huber, H. Asamer, H. Huber, H. Wigzell and H. Braunsteiner, Zur funktionellen Differenzierung lymphatischer Zellen. Lymphozyten mit Ig-Determinanten und Bindungsfähigkeit für Immunkomplementkomplexe bei der Maus. *Z. ges. exp. Med.* **156,** 34 (1971).

14. Ch. Huber, E. Dworzak, U. Fink, G. Michlmayr, H. Braunsteiner and H. Huber, Receptor sites for aggregated gammaglobulin (AGG) on lymphocytes in lymphoproliferative diseases. *Brit. J. Haematol.* (In press).
15. C. J. Elson, J. Singh and R. B. Taylor, The effect of capping by anti-immunoglobulin antibody on the expression of cell surface immunoglobulin and on lymphocyte activation. *Scand. J. Immunol.* **4,** 143 (1973).
16. M. Edidin and A. Weiss, Antigen cap formation in cultured fibroblasts; A reflexion of membrane fluidity and of cell motility. *Proc. nat. Acad. Sci.* **69,** 2456 (1972).
17. H. D. Flad, Ch. Huber, H. Bremer, H. D. Menne and H. Huber, Impaired recirculation of B lymphocytes in chronic lymphocytic leukaemia. *Europ. J. Immunol.* **3,** 688 (1973).

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In Vitro Demonstration of Cellular Hypersensitivity to Tumour Antigens by Means of the Leucocyte Migration Technique in Patients with Renal Carcinoma*

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Abstract—*Twenty-four patients with renal carcinoma, 6 patients with benign, solitary renal cysts, and 46 control patients were examined for tumour-directed, cellular hypersensitivity by means of the capillary tube leucocyte migration technique (LMT). The patients were tested with soluble extracts of: (1) foetal, human kidney; (2) allogeneic hypernephroma tissue and corresponding normal, renal tissue; (3) autologous renal tumour tissue and corresponding normal, renal tissue. No significant stimulation or inhibition of leucocyte migration was found in patients with renal cysts. Thirteen out of eighteen and 12 out of 23 patients with renal carcinoma showed a state of cellular hypersensitivity towards the extracts of hypernephroma and foetal renal tissue. This reactivity was demonstrated preoperatively in 6 out of 11 and 5 out of 13 patients respectively. Tissue preparations from adult normal renal tissue did not cause any reaction.*

Reactivity was found equally often in patients with and without regional lymph node metastases. Patients having distant metastases at the time of initial treatment, significantly less often had tumour-directed, cellular hypersensitivity than the patients without distant metastases.

INTRODUCTION

IN PATIENTS with renal tumours tumour-directed, cellular hypersensitivity can be demonstrated *in vitro* by various techniques in which the patients' lymphocytes are tested against autologous or allogeneic tumour cells. Such studies comprise the colony inhibition technique [1], the micro-assay target cell destruction [2–4] and the lymphocyte transformation test [5, 6]. Conflicting results have been reported concerning the correlations between tumour-directed, cellular hypersensitivity as measured *in vitro* and the clinical stage of the patients tested [7–11]. Cellular hypersensitivity to plated foetal gut and liver epithelial cells, and to foetal glial cells has been demonstrated in patients with colonic carcinomas [12] and brain tumours [13] indicating an onco-foetal, antigenic relationship.

Andersen *et al.*, [14, 15] were the first to demonstrate that the capillary tube leucocyte

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migration technique, (LMT) using peripheral leucocytes as migrating cells, and soluble tumour extracts as antigen, could be used for detection of tumour-directed, cellular hypersensitivity in man.

Since then, a number of reports using this technique in the study of tumour-directed, cellular hypersensitivity have appeared in the literature. The results of these studies have recently been reviewed in detail [16]. The purpose of the present investigation has been, by means of the LMT in patients with renal carcinomas:

(1) to study whether tumour-directed, cellular hypersensitivity towards allogeneic renal tumour extract could be detected in the preoperative period;
(2) to study the possible existence of a cross-reactivity between foetal renal tissue and renal tumour tissue;
(3) to study the possible correlations between tumour-directed, cellular hypersensitivity employing this technique and the clinical stage of the patients tested.

MATERIAL AND METHODS

The total number of patients in the material was 76 and comprised the following groups:

Twenty-four consecutive patients, 16 male and 8 female, aged 17–75, who entered the investigation when the diagnosis of renal carcinoma, had been rendered probably be intravenous urography or renal arteriography. Nine patients had demonstrable distant metastases. Fifteen patients had the histological diagnosis of hypernephroma. The rest of the material comprised nephroblastoma (3 patients), pelvic carcinoma (transitional cell carcinoma), (4 patients), and renal myxosarcoma (1 patient). One patient had no operation performed. In Table 1, the clinical data and tumour pathology of the 24 patients are presented.

Six patients, who had been operated upon because of benign solitary renal cysts.

Three patients with cancer of the lung.

Fourteen patients with cancer of the colon or rectum. The 17 cancer patients were without evidence of distant metastases.

Twenty-nine control patients tested with the same tissue extracts as the patients with renal carcinomas. Several control patients were tested repeatedly.

In this study a control patient is defined as a person without any subjective or objective signs of renal disease, malignant or benign tumours,

Table 1. *Patients with renal carcinomas. Clinical data, tumour pathology*

Patient		Sex	Age	Metastases at the time of initial treatment				Tumour type
				lymph nodes	distant			
					pulmonal	skeletal	other	
1	ETP	Female	71	0	0	0	0	Hypernephroma
2	CIB	Male	51	0	0	+	0	Hypernephroma
3	SAA	Male	68	0	0	+	0	Hypernephroma
4	NT	Male	61	+	0	0	0	Hypernephroma
5	SAN	Male	57	0	0	0	0	Malignant renal tumour
6	SGR	Female	17	+	0	0	0	Nephroblastoma
7	AT	Female	39	0	0	0	0	Hypernephroma
8	EF	Female	75	0	0	0	0	Pelvic carcinoma
9	KG	Male	68	+	0	+	0	Myxosarcoma
10	BKO	Male	16	0	0	0	+	Nephroblastoma
11	HJ	Male	68	0	+	0	0	Hypernephroma
12	JBJ	Male	60	0	0	0	0	Hypernephroma
13	BBL	Male	46	0	0	0	0	Pelvic carcinoma
14	AG	Female	63	0	0	0	0	Hypernephroma
15	JTH	Male	65	0	0	0	0	Hypernephroma
16	JUK	Male	71	0	0	0	0	Hypernephroma
17	LI	Male	61	0	(+)	0	0	Hypernephroma
18	IVP	Male	27	0	0	0	0	Pelvic carcinoma
19	HK	Male	62	+	0	0	0	Hypernephroma
20	ERH	Female	64	0	+	0	0	Hypernephroma
21	II	Female	51	+	0	0	0	Nephroblastoma
22	EMA	Female	57	0	0	+	0	Hypernephroma
23	RBA	Male	69	0	+	+	+	Hypernephroma
24	BUH	Male	44	0	0	0	0	Pelvic carcinoma

hypertension or autoimmune diseases, who has not received any treatment with steroids or cytostatic drugs during the last six months. Patients with the following diagnoses were included in the control group: pilonidal cyst [5]; diverticulosis of the colon [3]; duodenal ulcer [6]; cholelithiasis [3]; multiple sclerosis [1]; oesophageal stenosis [3]; oophoritic cyst [1]; sequelae after gastric resection [2]; inguinal hernia [1]; haemorrhoids [1]; chronic bronchitis, emphysema [1]; and healthy persons [2]. The control material comprised 20 male and 9 female patients, aged 20–71.

Preparation of tissue extracts

After removal of the kidney, specimens were obtained from tumour-tissue and from normal tissue not macroscopically invaded by tumour. This was performed aseptically, and always within 30 min after removal of the kidney. The tissue specimens were placed at −20°C.

On the day of operation or the day after, the specimens were thawed, cut into small pieces, suspended in Hanks' solution, and homogenized in a M.S.E. homogenizer at 15,000 rev/min for 10 min. The homogenates were kept at 4°C overnight, and then centrifuged at 1000 rev/min for 20 min.

The protein concentration of the supernatant was determined by Lowry's method, and adjusted to 1 mg per ml by dilution with Hanks' medium. Antibiotics were added, penicillin G, 1000 i.u. per ml, and streptomycin, 15 μg per ml. Culture reports showed that all tissue extracts were sterile. Two and a half ml aliquots of the extracts were transferred to sterile glass ampoules, and lyophilized. Immediately before use in the test the extracts were dissolved in the proper volume of sterile water. Using the same procedure, extracts were prepared from pooled, human foetal kidneys. The foetuses were all spontaneous or therapeutic abortions in the III trimester of gestation, without detectable malformations.

Concentration and types of tissue extracts used

The maximal concentration of tissue extract not causing unspecific (toxic) stimulation or inhibition of migration of normal leucocytes (maximal non-toxic dose) was determined for each extract by systematic titration experiments at 50, 100, 200, 300, 400, and 600 μg protein per ml.

The patients and controls were tested with the following types of tissue extracts in at least two concentrations:

(1) foetal kidney extracts—max non-toxic dose, 100 μg protein per ml.
(2) allogeneic hypernephroma extract and extract from the corresponding normal renal tissue—max non-toxic dose, 400 μg protein per ml.
(3) autologous tumour extract and extract from the corresponding normal renal tissue—max non-toxic dose, 400 μg protein per ml.

The leucocyte migration technique

The LMT was performed as described earlier by Bendixen and Søborg [17], with a few modifications.

On the day of testing, 40–80 ml of peripheral blood was collected from a cubital vein into 10 ml polysterol tubes containing 250 i.u. of heparin without preservatives, and 2·0 ml of 5% Dextran 250. The tubes were carefully inverted 10 times, and placed for 1 hr at 37°C.

After sedimentation of the erythrocytes, the leucocyterich supernatant was transferred to polyethylene tubes, and centrifuged at $1000 \times g$ for 5 min. The leucocytes were washed 3 times in Hanks' solution, and after resuspension in TC 199 with 10% horse serum, collected in a suitable number of siliconized glass capillary tubes. Each capillary tube had an internal diameter of 1·4 mm, and contained approximately 7×10^6 leucocytes consisting of approximately 40% lymphocytes and 60% granulocytes. After sealing one end in a flame and centrifuging at 2000 rev/min for 10 min, each capillary tube was cut just below the cell-fluid interfase, and placed in a 1·0 ml culture chamber containing TC 199 and 10% horse serum with a drop of silicone grease. The adequate volume of tissue extract was added, and the chamber was sealed with a microscope coverslip. Four culture chambers were set up for each concentration of tissue extract employed.

Within a set of identical cultures the variation from one migration to another did not exceed 10%. After 24 hr at 37°C, the migration area of the leucocytes around the opening of the capillary tube was measured in a projection microscope. The average migration area of the antigen-containing (M_x) and control cultures (M_0) from the same blood sample determine the migration index (MI):

$$MI - \frac{M_x}{M_0}$$

A MI of less than 1·0 indicates an inhibition and MI greater than 1·0 indicates a stimulation of leucocyte migration.

Test protocol

Three to eight tests (group of initial tests) were performed in each individual patient in

the pre- and immediate postoperative period, whenever possible employing all three types of tissue extracts. In the studies of the reactivity towards allogeneic tumour tissue and corresponding normal renal tissue, the patients were tested with non-pooled extracts from 2 to 6 different patients. The reaction was considered indicative of a state of cellular hypersensitivity when the patient's leucocytes were stimulated or inhibited by at least one of the tumour extracts used.

According to Søborg [18] both stimulation and inhibition of leucocyte migration on contact with specific antigen are indicative of a state of cellular hypersensitivity.

Statistical evaluation

For evaluation of the problem (whether two distributions according to MI had the same dispersion), the Fisher exact probability test was used, modification: the Westenberg interquartile range test [19]. The fourfold table contained, in the first line, the sum of observations in the upper and lower quartiles of the two groups compared, and in the second line, similar data for the two inner quartiles—level of significance: 5%. Moreover, the mean values ± 2 SD of the distributions of MI in the control groups were calculated to indicate an upper and a lower limit of the reactions. This was done in order to place single values of MI obtained in relation to what could be regarded as a "normal range", presupposing a normal distribution of the controls.

Normal ranges:

(1) foetal kidney tissue, 100 μg per ml = 0·78–1·18;
(2) hypernephroma extract, 400 μg per ml = 0·79–1·19;
(3) normal renal tissue extract, 400 μg per ml = 0·79–1·19.

RESULTS

The results are presented in the Figs. 1–5.

Reaction to autologous tumour extract

Two out of eleven patients with renal carcinomas had MI > 1·19, and 6 out of 11 had MI < 0·79 when tested with autologous tumour extract. No significant effect was found when leucocytes from renal carcinoma patients were tested towards extracts of autologous, normal renal tissue.

Reaction to pooled foetal renal tissue

None of the patients with renal cysts, carcinoma of the lung, and carcinoma of the colon or rectum exhibited MI's different from the normal range when tested with extracts of pooled foetal renal tissue (Fig. 1). Three out of twenty-three patients with renal carcinomas had MI > 1·18, and 9 out of 23 had MI < 0·78. One out of thirteen had a stimulation and 4 out of 13 an inhibition of leucocyte migration when tested towards foetal kidney extract preoperatively. Thus, a state of cellular hypersensitivity towards foetal kidney extract seems to be present in some patients with renal

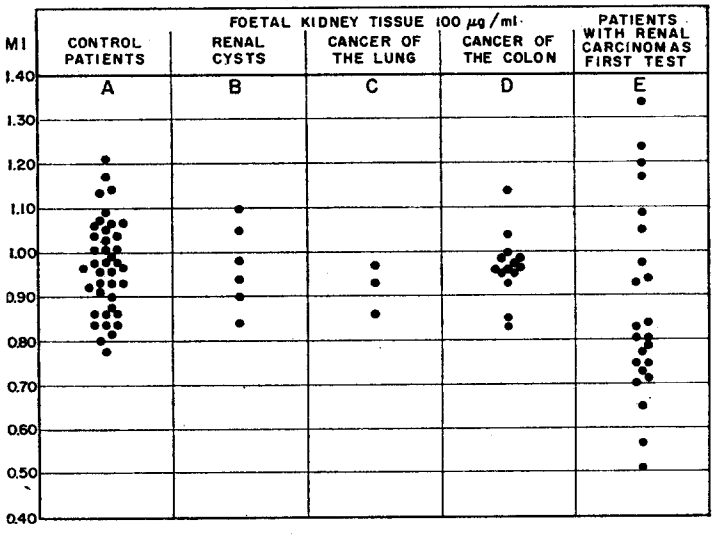


Fig. 1. Cellular hypersensitivity towards soluble extract of pooled foetal renal tissue, 100 μg protein per ml in control patients, column A, patients with solitary renal cysts, column B, patients with cancer of the lung, column C, patients with cancer of the colon, column D, and patients with renal carcinomas, column E.

Fischer's exact probability test: A–E, P < 0·001, *B–E,* P < 0·001. *D–E,* P < 0·001. *A–B, A–D,* P < 0·05.

carcinoma, although further specificity controls are required regarding the organ-specificity of the reactions. In the same patients, it was possible only rarely to detect a reaction of cellular hypersensitivity towards extracts of adult human renal tissue (Fig. 2, column D and F). No correlation was found between leucocyte stimulation or inhibition and the previous history of pregnancy or transfusion.

Cross-reactivity between renal carcinomas

In Fig. 2, the reactivity of leucocytes from renal carcinoma patients and control patients towards allogeneic hypernephroma tissue and corresponding allogeneic normal renal tissue is presented.

Two out of eighteen patients in the renal carcinoma group had MI > 1·19, and 11 out of 18 had MI < 0·79, indicating stimulation and inhibition of leucocyte migration respectively. Before operation, 1 out of 11 patients had a stimulation and 5 out of 11 patients had a inhibition of leucocyte migration. Fourteen out of fifteen patients with hypernephroma were tested with allogeneic hypernephroma extract. Migration indices indicating a state of cellular hypersensitivity were found in 10 out of 14 patients. Four patients with a histological diagnosis other than hypernephroma were tested with allogeneic hypernephroma extracts. One patient with a nephroblastoma, and 2 out of 3 patients with transitional cell carcinoma cross-reacted with these extracts. These patients were without distant metastases at the time of testing.

These findings seem to indicate that tumour-directed, cellular hypersensitivity towards allogeneic hypernephroma tissue can be detected in some patients with renal carcinoma. Only in a few tests reactivity was found, when the patients were tested with extracts of adult, normal renal tissue, and leucocytes from control patients also were unaffected by the tissue extracts employed.

Correlations between the clinical stage of the patients and the presence of tumour-directed, cellular hypersensitivity

At operation, 5 patients had histologically verified, regional lymph node metastases. Four out of five, 3 out of 3, and 3 out of 3 of these patients were reactive towards extracts of foetal kidney, allogeneic hypernephroma, and autologous tumour tissue respectively, compared to 8 out of 18, 10 out of 15, and 5 out of 8 respectively, in the group without regional lymph node metastases. The difference between the reactivity in the two groups was not significant ($P > 0{\cdot}05$).

In Figs. 3–5, the reactivity of the patients with and without clinically evident distant metastases at the time of operation towards foetal kidney tissue, allogeneic hypernephroma tissue and corresponding normal tissue is presented.

Employing both types of extracts tumour-directed, cellular hypersensitivity was found

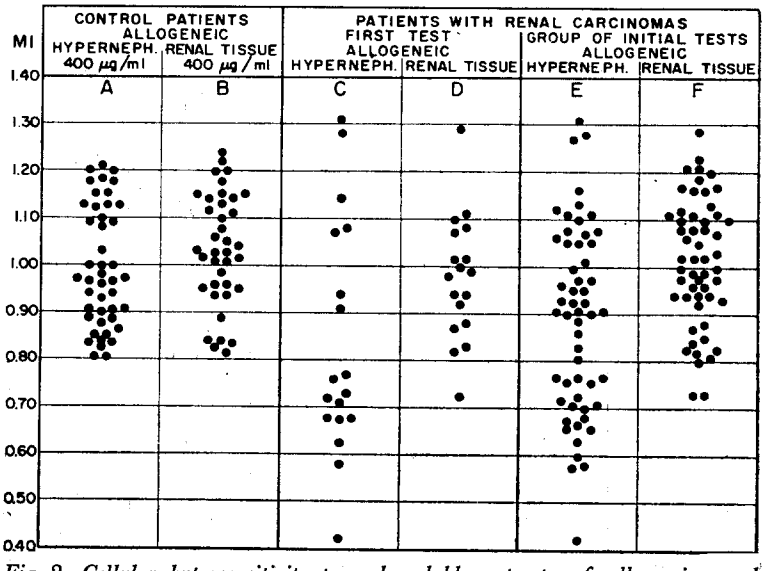


Fig. 2. Cellular hypersensitivity towards soluble extracts of allogeneic renal carcinoma tissue (hypernephroma) and normal renal tissue, 400 µg protein per ml in control patients, column A+B, and in patients with renal carcinomas, column C+D, first test performed in each patient, and column E+F, total number of tests performed in the period of initial treatment.

Fisher's exact probability test: A–B, P > 0·05, *C–D*, P < 0·01, *E–F*, P < 0·01. *A–C*, P < 0·01. *A–E*, P < 0·01. *B–D*, P > 0·05. *B–F*, P > 0·05.

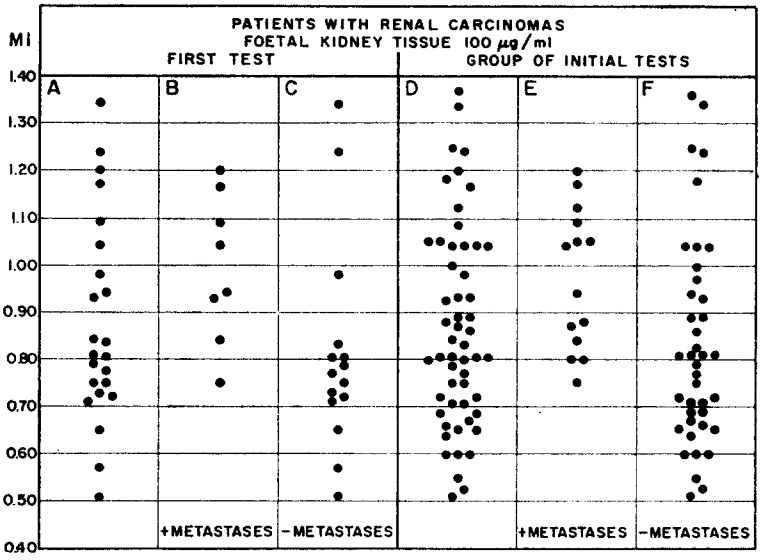


Fig. 3. Correlation between the presence of distant metastases at the time of initial treatment and, cellular hypersensitivity towards soluble extracts of pooled, foetal kidney tissue, 100 µg protein per ml in patients with renal carcinomas.
Fischer's exact probability test: B–C, $P < 0{\cdot}01$. *E–F,* $P < 0{\cdot}01$.

significantly less often in patients with, rather than in patients without, distant metastases.

DISCUSSION

Investigations concerning cellular hypersensitivity towards oncofoetal antigens in human tumours have been few. Hellström *et al.* [12] demonstrated that blood lymphocytes from patients with colonic carcinomas inhibited colony formation of plated colonic carcinoma cells and of epithelial cells from foetal gut and liver. The tissues employed were from I and II trimester foetuses. Similar results have been obtained in patients with malignant tumours of the brain [13], and carcinoma of the breast [20].

The results of the present study seem to be in accordance with those of Hellström *et al.* [12] apart from the fact that the foetal gut and liver antigens were present during the I and II trimester of gestation, while the kidney antigen(s) demonstrated in this study was present in the III trimester of gestation.

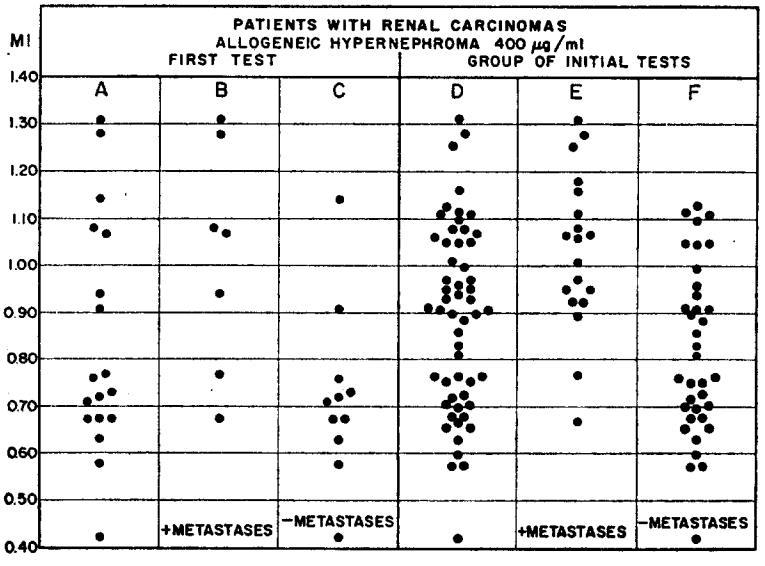


Fig. 4. Correlation between the presence of distant metastases at the time of initial treatment and tumour-directed, cullular hypersensitivity towards soluble extracts of allogeneic hypernephroma tissue, 400 µg protein per ml in patients with renal carcinoma.
Fischer's exact probability test: B–C, $P < 0{\cdot}01$. *E–F,* $P < 0{\cdot}01$.

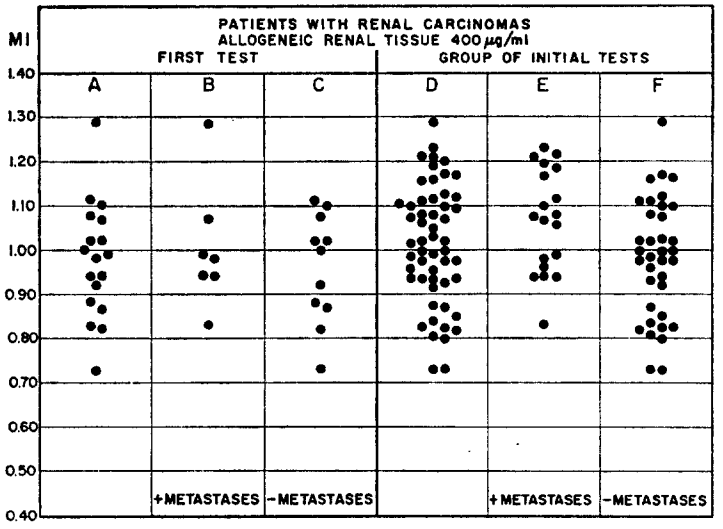


Fig. 5. Correlation between the presence of distant metastases at the time of initial treatment and tumour-directed, cellular hypersensitivity towards soluble extracts of allogeneic, adult, normal renal tissue, 400 µg protein per ml in patients with renal carcinomas.

Fischer's exact probability test: B–C, $P > 0{\cdot}05$. *E–F,* $P > 0{\cdot}05$.

At least two possiblities exist regarding the mechanism underlying the reactivity towards foetal kidney extract found in renal carcinoma patients:

(1) the reaction could be unspecific and a result of renal necrosis. This hypothesis is supported by the findings of similar reactivity in patients with glomerulonephritis [21, 22] and in kidney grafted patients [23].

(2) the reaction could be a result of a specific depression of foetal antigens in tumourous tissue [24]. The following finding supports this point of view: three types of tissue extracts were employed: (a) pooled foetal renal tissue, (b) allogeneic hypernephroma tissue, (c) autologous renal tumour tissue.

Four out of twenty-four patients were tested with one type of tissue extract, 12 out of 24 with two, and 8 out of 24 with all three types of tissue extracts employed as described above. Of those tested with two types of extracts, 3 out of 12 did not react with any, 3 out of 12 were reactive with one type, and 6 out of 12 were reactive towards both types of extracts on at least one occasion. Of the patients tested with three types of extracts, 2 out of 8 did not react with any, 2 out of 8 were reactive towards two, and 4 out of 8 reacted against all three types of tissue extracts. Of course, it could be postulated that all the reactions were due to necrosis, but in that case one should have expected reactivity towards renal tissue from adults. However, the reactions demonstrated were confined to the tumour extracts and the extracts from foetal kidney tissue. Variations in the total number of peripheral leucocytes could not explain the differences in reactivity those patients with a cellular hypersensitivity reaction having a mean value of 6000 leucocytes per µl consisting of 73% granulocytes (SD ± 14) and 26% lymphocytes (SD ± 11), compared to 5000, 65% (SD ± 13) and 34% (SD ± 12) in the non-reactive group. These differences are not significant ($P > 0{\cdot}05$).

It is generally accepted that patients with advanced malignant disease are often non-reactive when tested intracuteneously with battery of antigens [25, 26]. Likewise the ability of lymphocytes from cancer patients to transform in the presence of PHA has been shown to be decreased in case of disseminated tumour [27, 28]. Concerning tumour-directed, cellular hypersensitivity as measured *in vitro*, conflicting results have been reported [7–11]. However, when quantitative assays have been employed, correlations of hypersensitivity to the clinical stage of the patients have often been reported [3, 9, 10].

The data from the present study seem to indicate that when the first immunological barrier, the regional lymph nodes, has been overcome by the tumour, the ability to mount a reaction of tumour-directed, cellular hypersensitivity decreases significantly in patients with renal carcinomas. Currie recently demonstrated [29] that a factor coating the lymphocytes in patients with disseminated tumours

could be removed by intensive washing rendering the former non-reactive lymphocytes cytotoxic for the relevant target cells. Non-reactivity could be restored by including serum from patients with the same type of disseminated tumour in the test system.

It has been shown earlier [30] that preoperative serum from patients with hypernephroma could abolish tumour-directed, cellular hypersensitivity as measured in the present test system.

The results described above were obtained with leucocytes washed only three times. Preliminary observations indicate that increasing the number of washings to 6 may remove "non-reactivity" in patients with disseminated hypernephroma, and that "non-reactivity" may be restored by including autologous serum in the test system.

The implications of these observations are, at present, under active study.

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REFERENCES

1. I. Hellström, K. E. Hellström, H. O. Sjögren and G. A. Warner, Demonstration of cell-mediated immunity to human neoplasms of various histological types. *Int. J. Cancer* **7,** 1 (1971a).
2. J. Bubenik, J. Jakoubkova, P. Krakòra, M. Baresova, P. Helbich, V. Viklicky and V. Malaskova, Cellular immunity to renal carcinomas in man. *Int. J. Cancer* **8,** 503 (1971).
3. V. Diehl, B. Jereb, J. Stjernswärd, C. O'Toole and L. Åhström, Cellular immunity to nephroblastoma. *Int. J. Cancer* **7,** 277 (1971).
4. B. Jereb, V. Diehl, L. Åhström and I. Juhlin, Relation between cellular immunity to nephroblastoma and the phase of the disease. *Acta paediat. Scand.* **61,** 709 (1972).
5. J. Stjernswärd, Effect of autochthonous cancer on the immune response. In *Oncology*. Edited by R. E. Clark *et al.*, Vol. II, p. 322. Year Book Medical Publishers Inc., Chicago 1970.
6. J. Stjernsärd, In *Immunologic Intervention*, J. W. Uhr and M. Landy. Proc. Int. Conf., Brooke Lodge, Augusta, Michigan, April 26–28 (1971) pp.242—265. Academic Press, New York. (1971).
7. I. Hellström, K. E. Hellström, A. H. Bill, G. E. Pierce and J. P. S. Yang, Studies on cellular immunity to human neuroblastoma cells. *Int. J. Cancer* **6,** 172 (1970).
8. J. E. De Vries, P. Rümke and J. L. Bernheim, Cytotoxic lymphocytes in melanoma patients. *Int. J. Cancer* **9,** 567 (1972).
9. C. O'Toole, P. Perlman, B. Unsgaard, G. Moberger and F. Edsmyr, Cellular immunity to human urinary bladder carcinoma. I. Correlation to clinical stage and radiotherapy. *Int. J. Cancer* **10,** 77 (1972a).
10. C. O'Toole, P. Perlman, B. Unsgaard, L. E. Almgård, B. Johansson, G. Moberger and F. Edsmyr, Cellular immunity to human urinary bladder carcinoma. II. Effect of Surgery and preoperative irradiation. *Int. J. Cancer* **10,** 92 (1972b).
11. A. J. Cochran, R. M. Mackie, C. E. Thomas, R. M. Grant, D. E. Cameron-Mowat and W. G. S. Spilg, Cellular immunity to breast carcinoma and malignant melanoma. *Brit. J. Cancer* **28,** (Suppl. 1) 77 (1973).
12. I. Hellström, K. E. Hellström, T. H. Shepard, Cell-mediated immunity against antigens common to human colonic carcinomas and foetal gut epithelium. *Int. J. Cancer* **6,** 346 (1970).
13. T. Wahlström, E. Saksela and H. Troupp, Cell-bound antiglial immunity in patients with malignant tumours of the brain. *Cell. Immunol.* **6,** 161 (1973).
14. V. Andersen, G. Bendixen and T. Schiødt, An *in vitro* demonstration of cellular immunity against autologous mammary carcinoma in man. Preliminary report. *Acta med. scand.* **186,** 101 (1969).
15. V. Andersen, O. Bjerrum, G. Bendixen, T. Schiødt and I. Dissing, Effect of autologous mammary tumour extracts on human leucocyte migration *in vitro*. *Int. J. Cancer* **5,** 357 (1970).

16. V. ANDERSEN, M. KJÆR and G. BENDIXEN, *In vitro*-demonstration of cellular hypersensitivity to tumour antigens in man. *Ser. Haemat.* **5,** 3 (1972).
17. G. BENDIXEN and M. SØBORG, A leucocyte migration technique for *in vitro* detection of cellular (delayed type) hypersensitivity in man. *Dan. med. Bull.* **16,** 1 (1969).
18. M. SØBORG, *In vitro* migration of peripheral human leucocytes in cellular hypersensitivity. *Acta med. scand.* **184,** 135 (1968).
19. J. W. BRADLEY, *Distribution—Free Statistical Tests.* Prentice-Hall, Englewood Cliffs. N.J. New Jersey, 1968.
20. D. Della Porta, S. Canevari and G. Fossati, Immune response to and embryo cells in patients with mammary carcinoma. *Brit. J. Cancer* **28,** (Suppl. 1), 103 (1973).
21. G. BENDIXEN, Organ-specific inhibition of the *in vitro* migration of leucocytes in human glomerulonephritis. *Acta med. scand.* **184,** 99 (1968).
22. R. E. ROCKLIN, E. J. LEWIS and J. R. DAVID, *In vitro* evidence for cellular hypersensitivity to glomerular basement membrane antigens in human glomerulonephritis. *New Engl. J. Med.* **283,** 497 (1970).
23. E. WEEKE, B. WEEKE and G. BENDIXEN, Organ-specific, antirenal cellular hypersensitivity after kidney transplantation. *Acta med. scand.* **188,** 307 (1970).
24. P. GOLD, Embryonic origin of human tumour-specific antigens. *Progr. exp. Tumour Res.* **14,** 43 (1971).
25. M. J. KRANT, G. MANSKOPF, C. S. BRANDRUP and M. A. MADOFF, Immunologic alterations in bronchogenic cancer. Sequential study. *Cancer* **21,** 623 (1968).
26. F. R. EILBER and D. L. MORTON, Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer* **25,** 362 (1970).
27. D. B. GARRIOCH, R. A. GOOD, and R. A. GATTI, Lymphocyte response to P.H.A. in patients with non-lymphoid tumours. *Lancet* **i,** 618 (1970).
28. M. G. WHITTAKER, K. REES and C. G. CLARK, Reduced lymphocyte transformation in breast cancer. *Lancet* **i,** 892 (1971).
29. G. A. CURRIE and C. BASHAM, Serum mediated inhibition of immunological reactions of the patient to his own tumour: A possible role for circulating antigen. *Brit. J. Cancer* **26** 427 (1972).
30. M. KJÆR, The capillary tube leucocyte migration technique (LMT) as a method for detection of serum inhibiting activity of tumour-directed, cellular hypersensitivity in patients with renal carcinomas. *Acta path. microbiol. scand. Sect. B.* (In press).

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The Effect of Cell Dose and Distribution on the Development of a Transplanted Tumour*

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Abstract—*Experiments with an isogeneically-transplanted murine sarcoma which has a TD50 of about 30 cells have shown* inter alia *that for a given total cell dose the proportion of mice which develop tumours is not altered by distributing the dose among 10 sites as against concentrating it in a single site, whereas the tumour volume per mouse 24 days after inoculation may be markedly reduced. At doses of 100 cells per site or more the chance of take at a site is not influenced by the presence of other sites, but the mean rate of growth at a site is reduced in the presence of other sites.*

In T-cell deprived mice single site inoculation of 1000 cells results in slower growth than in intact mice, but distribution of the dose among multiple sites in deprived mice does does not further reduce the total tumour volume (measured 18 or 24 days after inoculation).

In the light of these and other findings it is postulated that, following tumour inoculation, there is an early local host reaction probably mediated by macrophages, which causes rapid destruction of some of the transplanted cells, the number destroyed depending partly on the intensity of the reaction and partly on a mutually protective effect of the tumour cells. With tumours which possess tumour-specific transplantation antigens there is in addition a slower immunological reaction which may destroy some or all of the cells which have survived the early reaction. A modification of the hypothesis put foreward previously to account for the slower growth of the tumour in T-cell deficient mice is proposed.

INTRODUCTION

THE TECHNIQUE of dilution assay has been used by Hewitt and others [1, 2, 3, 4] to determine the TD50 (i.e., the number of tumour cells required to produce 50% takes) and other information concerning the transplantation kinetics of tumour cells.

The interpretation of the TD50 and the calculation of its standard error or confidence limits is commonly based on three assumptions [4]:

1. Some of the tumour cells are clonogenic.
2. An inoculum produces a take if, and only if, it contains at least one clonogenic cell.
3. Any non-clonogens in the inoculum have no influence on the outcome (take or no take).

According to Porter *et al.* [4], many transplanted tumours do behave in a way consistent with these assumptions, and these are described as "transplanting by single cells". Some tumours, however, do not conform, and these are said to show "anomalous kinetics".

With tumours which show anomalous kinetics the usual type of analysis by Finney's method [5] may only slightly bias the estimate of the TD50 but, as Porter *et al.* [4] point out, may give badly misleading standard errors. These authors have therefore proposed another mathematical model which generates a family of curves relating the log number of cells injected and the proportion of takes, one member of which corresponds to the special

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case of "transplantation by single cells". Their choice of model was however based on considerations of convenience and mathematical simplicity, and the biological situation is far from clear even in the special case and *a fortiori* when the kinetics are clearly anomalous.

In the first place, the single cell model is not necessarily valid even if the behaviour of a tumour is apparently consistent with it, for as Finney [5] has remarked, "if inferences are to be drawn from data . . . in which the test of validity will be insensitive to all but the grossest discrepancies, the *a priori* evidence for the validity of the mathematical model must be strong". That this is by no means the case here is shown by studies of the so-called Révész [6] effect by Hewitt, Blake and Porter [7], in which it was shown that when lethally irradiated cells (which must surely be classed as non-clonogenic) are added to the inoculum, the TD50 may be reduced dramatically despite the fact that the tumour, both with and without the addition of such cells, apparently conforms to the single cell model.

Secondly, the high TD50 sometimes found with tumours which apparently conform to single cell kinetics (6900 in one CBA carcinoma studied by Hewitt, Blake and Porter [7]) is far in excess of the theoretical value of $\log_e 2$ (i.e. 0·693) to be expected if all viable cells in the inoculum were clonogens, and this would seem to cast doubt on the very concept of a cell being clonogenic in an absolute sense. The possibility that the vast majority of cells in the inoculum, though apparently viable by *in vitro* tests, were potentially incapable of giving rise to a tumour *in vivo*, not only seems implausible *a priori*, but would appear to be formally excluded by the observation that the addition of an appropriate number of irradiated tumour cells reduced the TD50 to about 4 viable cells.

Thirdly, in the case of tumours which clearly did not conform to the single cell model, analysis according to the extended method of Porter *et al.* [4] led to the extraordinary conclusion that the number of clonogens in the inoculum was proportional not to the total number of cells but to a fractional power of this number.

The present investigation was undertaken in the hope that some further understanding of the situation might be gained by comparing the effect of (a) injecting a given number of tumour cells into a single subcutaneous site, and (b) dividing the same number of cells between 10 different subcutaneous sites, using as recipients both normal and T-cell deficient mice.

MATERIAL AND METHODS

Ten groups of intact, and two groups of T-cell deprived, adult female CBA mice were set up as shown in Table 1. Mice of groups 7–10 inclusive were injected subcutaneously on one occasion with 0·05 ml of a suspension of viable tumour cells in phosphate buffered saline (PBS) at each of 10 sites located as shown in Fig. 1. Those in the other groups were given a

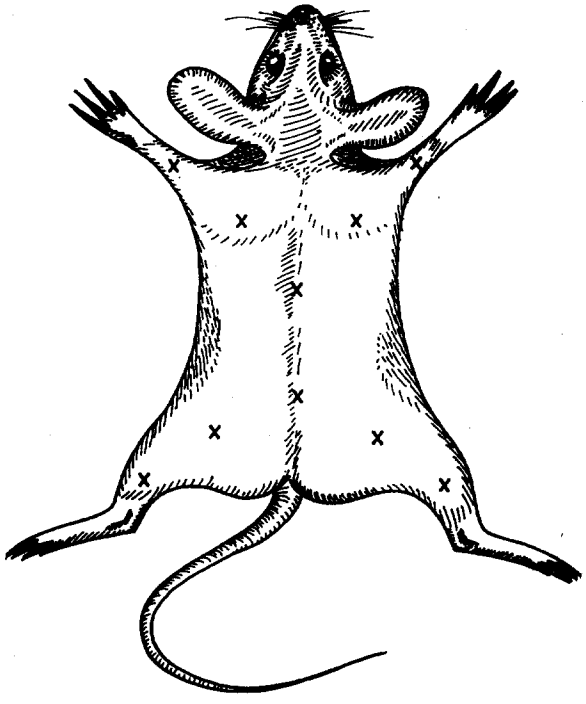


Fig. 1. Diagram showing sites for multiple tumour inoculations.

single subcutaneous injection of 0·1 ml tumour cell suspension to the right hind leg, and an injection of PBS to the other nine sites. The tumour cell suspension, which contained about 95% viable cells as judged by trypan blue exclusion, was diluted so that the mean number of viable cells injected at each site (ranging from 10 to 10,000) was as shown in the table. All the injections were made with a Hamilton 50 μl automatic syringe.

The cell suspensions were prepared as described by Woodruff, Inchley and Dunbar [8], and the T-cell deprived mice as described by Woodruff, Dunbar and Ghaffar [9]. The thymectomies were kindly performed by Dr. Ghaffar.

The mice were inspected every 2 days and when a tumour was present its mean diameter was measured in two directions at right angles with a caliper. The volume of each tumour was calculated on the assumption that it occupied a sphere whose mean diameter was equal to the mean of the two measured diameters. Mice with

Table 1. *Effect of cell dose and distribution on tumour development*

Group No.	Mouse intact or T-cell deprived	No. in group	Total No. of cells injected	No. of sites	Mean No. of cells per site	No. of mice with tumours			No. of sites with tumours		Mean diameter of tumours present (mm)		Mean volume of tumour per mouse with tumour (mm^3)		Mean volume of tumour per mouse in group (mm^3)	
						Day 18	Day 24	Day 48	Day 18	Day 24	Day 18	Day 24	Day 18	Day 24	Day 18 ± S.E.	Day 24 Vol ± S.E.
1	Intact	13	10	1	10	3	6	7	3	6	3·3	8·2	33	464	7·6 ± 5·5	215 ± 104
2	Intact	13	30	1	30	6	10	10	6	10	3·2	6·9	25	255	11·6 ± 5·7	196 ± 68
3	Intact	13	50	1	50	7	7	9	7	7	3·3	7·7	30	324	16·4 ± 7·0	174 ± 75
4	Intact	13	100	1	100	6	9	9	6	9	3·3	6·8	108	328	49·7 ± 32·2	227 ± 115
5	Intact	7	1000	1	1000	7	7	7*	7	7	8·1	15·0	327	1855	327 ± 74·6	1855 ± 287
6	Intact	7	10,000	1	10,000	7	7	7*	7	7	11·7	17·1	1023	2693	1023 ± 247	2693 ± 783
7	Intact	12	100	10	10	0	7	8	0	13	0	3·6	0	106	0	62 ± 45
8	Intact	7	1000	10	100	7	7	7	33	56	2·4	4·7	38	496	38·2 ± 7·3	496 ± 131
9	Intact	7	10,000	10	1000	7	7	7*	64	70	4·3	†	444	†	444 ± 130	†
10	Intact	7	100,000	10	10,000	7	7	7*	67	70	6·1	†	1395	†	1395 ± 303	†
11	Deprived	5	1000	1	1000	5	5	5*	5	5	5·4	9·0	118	515	118 ± 48	515 ± 198
12	Deprived	6	1000	10	100	6	6	6*	21	51	4·8	4·0	8·2	534	82 ± 19·6	534 ± 106

t-test comparison of group mean volumes

Groups compared	*t*	*P*
4 and 7 on Day 24		Not significant‡
5 and 8 on Day 24	4·316	0·001
5 and 11 on Day 24	3·511	< 0·01
6 and 9 on Day 18	2·075	just 0·05

*Includes tumour bearing mice which had been killed between Day 24 and Day 48.
†Incomplete because some mice were killed before Day 24 on account of ulcerating tumours.
‡Standard *t*-test not valid because of high variance ratio but means do not differ significantly when tested by the Behrens distribution ($d = 1{\cdot}34; -\tan\theta = 0{\cdot}390$).

single tumours were killed when the tumour diameter was about 2 cm, or earlier if the tumour began to ulcerate; those with multiple tumours when any had begun to ulcerate or appeared to interfere with normal activity of the mouse.

RESULTS

The results are summarised in Table 1. Analysis of the data points to the following conclusions:

1. The TD50 in intact mice for single site inoculation of the tumour by Finney's method is about 27 cells.

2. The transplantation kinetics are anomalous according to the criteria of Porter *et al.* [4]. This is suggested by inspection of the table, which shows more takes than would be expected from the single cell model at low dosage (10 cells) and fewer than expected at moderately high dosage (100 cells), and confirmed by calculation ($\chi^2 = 19{\cdot}5$ for 3 d.f.; $P < 0{\cdot}001$).

3. For a given total cell dose (at the levels studied) the proportion of mice which develop tumours is not altered by distributing the dose equally between 10 sites as compared with concentrating it at a single site.

4. At doses of 100 cells per site or more the chance of take at a site is not influenced by the presence of other sites, but at a dose of 10 cells per site the chance is reduced by the presence of other sites.

5. For a total tumour cell dose of 1000 cells or more the tumour volume per mouse 18 or 24 days after inoculation is less when the dose is distributed among 10 sites than when it is concentrated at a single site.

6. For a given site dose the mean rate of growth at the site is less if other sites have been inoculated.

7. For single site injection the mean rate of growth is essentially the same for doses of 10 cells to 100 cells, but for higher doses it increases with the dose.

8. Single site injections of 1000 cells results in slower growth in T-cell deprived mice than in intact mice.

9. In T-cell deprived mice the volume of tumour per mouse 18 or 24 days after injection is the same when a total dose of 1000 cells is distributed between 10 sites as when it is concentrated at a single site, and is about the same as in intact mice which received 1000 cells divided between 10 sites.

DISCUSSION

In the light of our own observations and those of others cited in the introduction we would propose the following hypotheses:

1. With all transplanted isogeneic tumours there is an early local host reaction, probably mediated by macrophages, which causes rapid destruction of some of the transplanted cells. The number of cells destroyed depends partly on the intensity of the host reaction and partly on a mutually protective effect of the tumour cells, the magnitude of which increases with the number of cells transplanted up to about the TD50 and then remains fairly constant.

2. With tumours which possess tumour specific transplantation antigens (TSTA) there is in addition a slower immunological reaction which may (a) destroy any tumour cells which have survived the early local reaction and so result in failure to take, or (b) retard the growth of the tumour when the number of cells inoculated is sufficient to ensure a take.

All except one of our findings seem to be not only consistent with, but readily accounted for by, the above hypotheses.

The tumour is known to possess TSTA because injection of irradiated cells prevents take following inoculation of 10^6 viable cells two weeks later [9]. The anomalous kinetics can be accounted for by mutual protection operating to increase the number of takes at low dosage but not increasing sufficiently with increasing dosage to compensate for the increased immunological reaction at intermediate dose levels, i.e. up to the level at which take occurs in virtually all animals.

Distribution of the inoculum among multiple sites in intact animals might be expected to facilitate immunization, especially with immunologically suboptimal doses, and in consequence to result in either a smaller proportion of takes or, as we have observed, slower growth of the tumour. It would also be expected, and again our observations have shown, that for a given site dose, growth at the site in question would be slower in intact mice, but not in T-cell deficient mice, in the presence of other sites of inoculation.

On the other hand our hypotheses do not explain the fact that single site injection of the same number of cells causes slower growth in T-cell deficient mice than in intact mice. This finding is consistent with the results reported in a previous paper [9], but we have as yet no evidence to confirm or refute the explanation which we then suggested, namely, that lack of T-cell helper function may diminish the produc-

tion of blocking antibody (or antibody capable of combining with tumour antigen to form blocking antigen-antibody complexes) to an extent which outweighs any impairment of resistance to the tumour directly attributable to the T-cell deficiency. A possible, but as yet speculative, modification of this hypothesis which we and our colleagues (Dr. K. James, Dr. A. Ghaffar and Dr. W. McBride) are investigating is that T-cell deficient mice may produce effective amounts of opsonising antibody but differ from intact mice in that they are unable to produce competitive non-opsonising antibody.

Another question of considerable interest to which as yet no complete answer can be given concerns the number of cells from which a tumour arises in relation to the number of cells injected. The fact that the growth rate was similar after single site injection of 10 or 100 cells, but for higher doses increased with the dose, at least raises the possibility that after inoculation of 10 or 100 cells the tumours which occurred developed from a single cell, whereas after larger doses the tumours developed from more than one cell. The type of experiment described in this paper is not qualified to settle this question, but the suggestion might gain some strength if tests with other tumours, including some with a TD50 of a few thousand or more cells, showed that as a general rule the growth pattern was essentially the same for tumours which developed after inocula up to a little above the TD50 and that thereafter growth rate increased with increasing doses.

Further study of the kinetics of transplantation of tumours by cell suspensions might be expected to provide additional evidence either consistent with, or capable of refuting, our hypotheses. It seems possible that it might also provide useful guidelines from which to develop a model for the much more complex phenomenon of tumour metastasis.

REFERENCES

1. H. B. Hewitt and C. A. Wilson, A survival curve for mammalian leukaemia cells *in vivo*. *Brit. J. Cancer* **13,** 69 (1959).
2. R. J. Berry and J. R. Andrews, Quantitative studies of radiation effect on cell reproductive capacity in a mammalian transplantable tumour *in vivo*. *Ann. N.Y. Acad. Sci.* **95,** 1001 (1961).
3. H. S. Reinhold, *Stralingsgevoeligheid van Tumoren*. Thesis, Rotterdam (1967).
4. R. H. Porter, H. B. Hewitt and Eileen R. Blake, The transplantation kinetics of tumour cells. *Brit. J. Cancer* **27,** 55 (1973).
5. D. J. Finney, *Statistical Method in Biological Assay*, 2nd Edn. Charles Griffin, London (1964).
6. L. Révész, Effect of tumour cells killed by X-rays upon the growth of admixed viable cells. *Nature (Lond.)* **178,** 1391 (1956).
7. H. B. Hewitt, E. Blake and E. H. Porter, The effect of lethally irradiated cells on the transplantability of murine tumours. *Brit. J. Cancer* **28,** 123 (1973).
8. M. F. A. Woodruff, M. P. Inchley and Noreen Dunbar, Further observations on the effect of *C. parvum* and anti-tumour globulin on syngeneically transplanted mouse tumours. *Brit. J. Cancer* **26,** 67 (1972).
9. M. Woodruff, Noreen Dunbar and A. Ghaffar, The growth of tumours in T-cell deprived mice and their response to treatment with *Corynebacterium parvum*. *Proc. roy. Soc. Lond. B.* **184,** 97 (1973).

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The Experimental Antitumour Properties of Three Congeners of the Acridylmethanesulphonanilide (AMSA) Series*

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Abstract—*Varying dosage schedules of three AMSA congeners (4′-[(9-acridinyl)-amino] methanesulphon-m-anisidide; NSC156303, 4′-[(4-methyl-9-acridinyl)amino] methanesulphon-m-anisidide; NSC150440, 4′-[(4,5-dimethyl-9-acridinyl)amino] ethanesulphon-m-anisidide; NSC159862) were administered i.p. to (C_3H/HeJ × DBA_2/J)F_1 hybrids bearing L1210 leukaemia implanted at various sites (i.p., i.v., s.c., i.c.). Cytosine arabinoside was included in the experiments as a positive control agent.*

All AMSA agents could eradicate a L1210 population (10^5) in a high percentage of the animals if cancer cells had also been administered i.p.; there were few long-term survivors if tumour had been inoculated i.v.; there were fewer still if tumour had been grafted s.c. and there were no survivors when the leukaemia had been implanted i.c. Only the two more lipophilic methyl substituted AMSA congeners and cytosine arabinoside could significantly extend life of mice with i.c. leukaemia.

The most polar of the agents, NSC156303, appeared schedule dependent being more effective against i.p. L1210 if dosed repeatedly at short time intervals (8 × 3 hr). There was no significant evidence of such schedule dependency with any of the agents against s.c. or i.c. implanted leukaemia. In animals with i.p. implanted L1210, the AMSA agents provided comparable life extensions to cytosine arabinoside when this agent was administered at its optimum dose and schedule.

The AMSA variants significantly inhibited the growth of first generation transplants of C_3H/HeJ spontaneous mammary tumours.

One agent, NSC156303, has been examined in the B16 malignant melanoma and P388 leukaemia assays and produced a percentage of long-term survivors in both systems.

INTRODUCTION

IN THE COURSE of a study of the structure–antitumour relationships in a series of bis-quaternary salts [1], we discovered a new agent which showed excellent activity in L1210 screening systems [2]. Further development of this lead molecule (4′-[(9-acridinyl)amino] methanesulphonanilide; abbreviated AMSA) has provided a large number of agents evincing high activity in L1210 screening tests. The researches described below were initiated in an attempt to find: (a) if real differences in activity could be discerned between structurally similar congeners of the AMSA series, (b) what role physicochemical properties play in the biologic activities recorded.

For a preliminary examination three agents were selected, the generic formula for these compounds is shown in Fig. 1.

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In order to cure advanced leukaemia it is necessary that agents can reach all tumour cells in lethal concentration regardless of the anatomic location of those cells [3]. To appraise the ability of the AMSA agents to reach anatomically remote tumour cells we have measured the life extensions attainable when various dosage schedules of the drugs were administered intraperitoneally (i.p.) to mice with L1210 implanted at different sites.

The rate of movement of drug from the point of administration to the ultimate site of action is normally controlled by partition dependent processes and therefore the lipophilic–hydrophilic balance of the agent [4]. The differing abilities of the AMSA agents to influence remotely implanted depots of L1210 have been discussed in terms of the lipophilic–hydrophilic balance of the agents.

MATERIAL AND METHODS

Agents

The members of the AMSA series examined were prepared in this laboratory by the general methods evolved earlier [2]; full details of synthesis, physical constants, etc., will be published elsewhere. Drug Research and Development (D.R.D.), Chemotherapy, American National Cancer Institute (N.C.I.) has allotted NSC numbers as follows:

$R^1 = R^2 = R^3 = H$ (Fig. 1) NSC156303

$R^1 = CH_3, R^2 = R^3 = H$ NSC150440

$R^1 = R^2 = R^3 = CH_3$ NSC159862

Dissociation constants and partition coefficients were measured by ultraviolet spectroscopy [5, 6].

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine; NSC63878) was generously donated by Upjohn Pty., Ltd.

Animals

Mice were bred in the colonies of this laboratory and are progeny from nuclei stock of the DBA_2/J and C_3H/HeJ strains (Jackson Laboratories, 1971).

L1210 assays

Sublines of L1210 were received at three monthly intervals from D.R.D., N.C.I. through the courtesy of Dr. I. Wodinsky, Life Sciences Division, Arthur D. Little Inc.

L1210 assays were carried out in C_3H/HeJ female × DBA_2/J male F_1 hybrids. Animals of initial weight 18·5–22·5 g were used and these received counted inocula of L1210 cells at various sites either intraperitoneally; subcutaneously above the right axillar; intravenously through a tail vein or intracerebrally through a temporal fissure. Drugs were administered in water at a constant volume of 0·01 ml/g body weight. At the concentrations required true solutions of all agents were possible except for NSC159862 which, at the highest levels required for single dosing, had to be administered as a suspension in water. Control groups were injected with water alone (0·01 ml/g). There were ten animals in each group at a given drug dose level and there was one control group for every five test groups. Dose levels were separated by 0·18 log dose intervals. Initial testing examined dosage levels which ranged from the clearly toxic to the inactive; on repetition only the originally observed optimum and those doses bracketing the optimum dose were used. All treatment of L1210 groups started 24 hr after tumour inoculation and, unless otherwise stated, dosage was by the i.p. route.

Cytosine arabinoside was used as a positive control in all L1210 tests.

Animal deaths were recorded twice daily.

C_3H Mammary tumour assays

Spontaneous mammary tumours from retired C_3H/HeJ female breeding stock were excised and *ca.* 2 mm cubes were implanted s.c. by trocar into C_3H/HeJ female mice. Tumour dimensions were measured by caliper twice weekly; tumour volumes being calculated as $4/3\pi ab^2$ where a and b are major and minor semiaxes respectively [7]. From a group of animals that had received transplants from a single donor tumour those with regular shaped tumours of dimensions between 6 × 6 mm and 8 × 8 mm ($a \times b$) were selected. These selected animals were randomised into groups of eight. One group in six was retained as a control and remaining groups received the various drug doses as indicated.

RESULTS

Physicochemical parameters

The physicochemical properties of interest were the basic dissociation constants and the partition coefficients. It proved impossible to directly measure the pK_a values of the three AMSA agents in water for two reasons: (1) there was complicating ionisation of the sulphonamide function* at the pH's necessary to remove the proton from the acridinium salts; (2) the extreme water insolubility of the neutral forms of the three AMSA derivatives made it

impossible to obtain solutions over the range of pH values of interest. While pK_a' figures could have been measured in solvent–water systems, the unknown solvent corrections necessary make the pertinence of such measurements questionable [5]. In order to estimate dissociation constants, the pK_a of a simple AMSA analog (4′-[(9-acridinyl)amino]aniline) was measured (pK_a 8·8). This weakly tumour active agent [2] does not have a complicating sulphonamide component and proved sufficiently water soluble for pK determinations. It was assumed that substituents other than those attached directly to the acridine ring made little contribution to pK_a. To the pK_a for the basic 9-anilinoacridine system were then added the ΔpK_a figures recorded in the 9-aminoacridine series [8] for 4-methyl (+0·2) and 4,5-dimethyl (−0·6) substituents. This provided the pK_a estimates listed in Table 1.

Table 1. *Physicochemical parameters of the AMSA agents*

Agent	Estimated pK_a	Log *P n*-OctOH—0·01 N.HCl
NSC156303	8·8	0·03
NSC150440	9·0	0·15
NSC159862	8·2	0·32

As a measure of lipophilic–hydrohilic balance the logarithm of the partition coefficient in *n*-octanol—0·01 N.HCl was used [6]; the acidic aqueous phase ensuring that distribution of cationic species was measured. Hansch's demonstration of additivity of log *P* (π) substituent values [9] requires that log *P* for neutral drug species should be displaced from that measured for the cation (Table 1) by a constant factor (3·9) [6].

The methyl groups of NSC150440 and NSC159862, placed adjacent to the polar cationic centre, do not make their normal contribution ($\pi CH_3 = 0{\cdot}52$) [10] to overall log *P*. Neighbouring polar functions are known to modulate the π values of substituents [11].

Agent formulation

Originally the 3 AMSA agents were prepared as hydrochloride salts but these proved insufficiently water soluble. Screening results obtained with suspensions of the sparingly soluble hydrochloride salts were variable and the optimum doses and life extensions recorded varied quite widely between tests. Variations in screening results have previously been noted between suspensions and solutions of an epipodophyllotoxin derivative [12]. As the methanesulphonate salts the AMSA agents listed (Fig. 1) proved sufficiently water soluble to be administered as true solutions; screening results obtained with such solutions were readily reproduced.

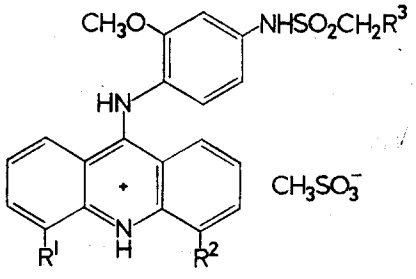


Fig. 1. *Generic structure of the AMSA congeners.*

*Schedule dependency against i.p. L*1210

The more polar of the AMSA derivatives (NSC156303) showed dosage schedule dependency [12] when assayed against early i.p. L1210 (Table 2). Dosing every 3 hr over a 24 hr period provided greater life extension than did a single optimum dose and an appreciable number of 50 day survivors were obtained. Toxic effects following the administration of this agent at the optimum single dose, or the maximum tolerated dose on a 3 hr × 8 schedule, persisted for greater than 4 days. If dosing was repeated at 4 day intervals lower doses were permissible and there was no overall gain in life extensions. The doses used for single day therapy could be repeated 8 days later without additional toxicity and then provided moderate increases in median survival times.

As the lipophilic character of the AMSA agents increased there was less evidence of dosage schedule dependency; the most lipophilic derivative (NSC159862) providing comparable life extensions and numbers of 50 day survivors with a variety of dosage schedules (Table 2).

As a chemotherapeutic index of use in assessing activity in L1210 tests the ratio of the optimum dose to that producing 40% increase in life survival (ILS_{40}) has been proposed [13]. ILS_{40} figures were derived from plots of life extension versus the logarithm of the dose [13]. From those experiments where dosing was once daily for 10 days the ratios optimum dose/ILS_{40} were 6·3 (NSC156303); 6·6 (NSC150440) and 6·8 (NSC159862). There is no significant difference between these ratios, but they demonstrate that broad ranges of doses provide significant tumour inhibitory effects.

*Measured pK_a methanesulphonanilide 8·9; 4-aminomethanesulphonanilide 9·3.

Table 2. Influence of treatment schedule on the activity of i.p. administered AMSA agents against i.p. L1210 (10^5)

Agent	Treatment schedule	Dose range (mg/kg per injection)	Optimum dose (mg/kg per injection)	Median survival times (days)		T/C%
				Treated	Control	
NSC156303	Daily, days 1–10	0·26–15	4·4	21	10	210
	Day 1 only	6·0–45	20	18	10	180
	Every 3 hr day 1	2·0–10	4·4	26(3)	10	260(3)
	Days 1, 5 and 9	6·0–30	13·4	18·5	9·5	195
	Every 3 hr days 1, 5 and 9	1·3–10	3·0	21	10·5	200
	Days 1 and 8	6·0–45	20	24	10	240
	Every 3 hr days 1 and 8	2·0–10	4·4	29(4)	10	290(4)
NSC150440	Daily, days 1–10	0·26–11	4·9	22(1)	9·5	232(1)
	Day 1 only	4.0–100	30	18	10	180
	Every 3 hr day 1	0·9–9·0	3·0	24(4)	10	240(4)
	Days 1, 5 and 9	6·6–45	20	23(1)	9·5	242(1)
	Every 3 hr days 1, 5 and 9	0·6–9·0	2·0	19	9·5	200
	Days 1 and 8	6·0–67	30	25	10	250
	Every 3 hr days 1 and 8	0·9–6·0	3·0	29(4)	10	290(4)
NSC159862	Daily, days 1–10	1·2–45	20	22(2)	9·5	232(2)
	Day 1 only	39–293	130	21(1)	10	210(1)
	Every 3 hr day 1	3·5–40	12	24(5)	10	240(5)
	Days 1, 5 and 9	26–196	87	29(2)	10	290(2)
	Every 3 hr days 1, 5 and 9	3·5–40	8	22(1)	10	220(1)
	Days 1 and 8	39–196	130	24(1)	10·5	228(1)
	Every 3 hr days 1 and 8	3·5–40	12	27(4)	10	270(4)
NSC63878 (Cytosine arabinoside)	Daily, days 1–10	10–79	35	18	9·5	189
	Day 1 only	2000–6700	3000	14	10	140
	Every 3 hr day 1	6·5–50	22	16	9·5	168
	Days 1, 5 and 9	670–4500	2000	19	9·5	200
	Every 3 hr days 1, 5 and 9	6·7–34	15	23(3)	10	230(3)

Tumour was implanted on day 0 and treatment was started 24 hr later (Day 1). There were 10 animals per dose level. Dose levels were separated by 0·18 log dose intervals.

The number of animals treated at the optimum dose who survived 50 days after the end of dosing are given in parentheses. Fifty day survivors have been ignored when calculating T/C.

Although our L1210 assays utilized C_3H/DBA_2F_1 hybrid mice rather than the more usual BDF_1 or CDF_1 [14] the life extensions recorded with cytosine arabinoside (Tables 2, 4) were in good accord with those provided by other authors [14–16]. The life extensions obtained with the AMSA variants appear comparable to those obtained with cytosine arabinoside in early i.p. L1210 tests.

A cytosine arabinoside resistant subline L1210/63878† appeared fully sensitive to the AMSA congeners (Table 3).

Drug effectiveness against remotely implanted L1210

The life extensions obtained when the AMSA agents were administered i.p. to mice with L1210 implanted at various sites (intravenously, subcutaneously and intracerebrally; i.v., s.c., i.c.) are recorded in Table 4. There was a clear attenuation of the life extensions recorded with the AMSA drugs and cytosine arabinoside as the separation between site of drug administration and tumour implantation increased (Fig. 2).

Table 3. Effectiveness of the AMSA congeners against cytosine arabinoside sensitive and resistant L1210

Agent*	Dose mg/kg per injection	L1210 T/C%	L1210/63878† T/C%
NSC156303	4·4	210	205
NSC150440	4·9	232(1)	215(2)
NSC159862	20	232(2)	210(3)
NSC63878‡	35	189	NS§

Mice received 10^5 L1210 cells i.p. on day 0.

*All agents were administered once daily i.p. on days 1–10.

†Cytosine arabinoside resistant L1210.

‡Cytosine arabinoside.

§Life extension not significant T/C < 125%.

†We are indebted to Dr. I. Wodinsky of Arthur D. Little Inc., for this L1210 subline.

Table 4. Effect of i.p. administered AMSA agents on survival times in mice inoculated with L1210 (10^5) at varying sites

Agent	Treatment schedule	Tumour site	Dose range (mg/kg per injection)	Optimum dose (mg/kg per injection)	Median survival times (days)		T/C%
					Treated	Control	
NSC156303	Daily, days 1–10	i.v.	1·9–6·6	4·4	18	11	164
		s.c.	1·9–6·6	4·4	15	11	137
		i.c.	1·9–6·6	4·4	10	9	NS
	Day 1 only	i.v.	9·0–30	20	17	10·5	162
		s.c.	9·0–30	20	14·5	11	132
		i.c.	9·0–30	20	9	9	NS
	Every 3 hr day 1	i.v.	2·0–6·6	4·4	19	11	173
		s.c.	2·0–6·6	4·4	15	10·5	143
		i.c.	2·0–6·6	4·4	10	9	NS
	Days 1, 5 and 9	i.v.	6·0–20	13·4	17·5	10·5	167
		s.c.	6·0–20	13·4	16	11	146
		i.c.	6·0–20	13·4	10	9	NS
	Every 3 hr days 1, 5 and 9	i.v.	1·3–4·5	3·0	16	11	146
		s.c.	1·3–4·5	3·0	15	11	137
		i.c.	1·3–4·5	3·0	10	9·5	NS
NSC150440	Daily, days 1–10	i.v.	2·2–7·5	5·0	19	10	190
		s.c.	2·2–7·5	5·0	16	11	146
		i.c.	2·2–7·5	5·0	12	9	133
	Day 1 only	i.v.	8·9–45	30	17	10	170
		s.c.	8·9–45	30	15	11	136
		i.c.	8·9–45	30	12	9·5	126
	Every 3 hr day 1	i.v.	1·3–4·5	3·0	20(1)	10	200(1)
		s.c.	1·3–4·5	3·0	16	11	145
		i.c.	1·3–4·5	3·0	11	9	NS
	Days 1, 5 and 9	i.v.	8·9–30	20	18(1)	10	180(1)
		s.c.	8·9–30	20	17	11	154
		i.c.	8·9–30	20	13	9	144
	Every 3 hr days 1, 5 and 9	i.v.	0·9–3·0	2·0	19	10·5	181
		s.c.	0·9–3·0	2·0	18	12	151
		i.c.	0·9–3·0	2·0	12	9	133
NSC159862	Daily, days 1–10	i.v.	8·9–30	20	23(1)	11	208(1)
		s.c.	8·9–30	20	17	11·5	148
		i.c.	8·9–30	20	13	9	144
	Day 1 only	i.v.	57–195	130	17·5	10·5	167
		s.c.	57–195	130	17	12	142
		i.c.	57–195	130	12·5	9	139
	Every 3 hr day 1	i.v.	5·3–18	12	22(1)	11	200(1)
		s.c.	5·3–18	12	18	12	150
		i.c.	5·3–18	12	12	9	133
	Days 1, 5 and 9	i.v.	39–130	87	26(3)	11	236(3)
		s.c.	39–130	87	21	11·5	183
		i.c.	39–130	87	14	9	155
	Every 3 hr days 1, 5 and 9	i.v.	5·3–18	8	20(1)	11	182(1)
		s.c.	5·3–18	8	19	12	158
		i.c.	5·3–18	8	12	9	133

Table 4 continued

Agent	Treatment schedule	Tumour site	Dose range (mg/kg per injection)	Optimum dose (mg/kg per injection)	Median survival times (days) Treated	Control	T/C%
NSC63878 (Cytosine arabinoside)	Daily, days 1–10	i.v.	16–53	35	17	10	170
		s.c.	16–53	35	16	11	146
		i.c.	16–53	35	13	9	144
	Day 1 only	i.v.	1300–4500	3000	13·5	10	135
		s.c.	1300–4500	3000	14	11	127
		i.c.	1300–4500	3000	12	9	133
	Every 3 hr day 1	i.v.	10–33	22	16	10	160
		s.c.	10–33	22	15	10·5	143
		i.c.	10–33	22	12	9	133
	Days 1, 5 and 9	i.v.	900–3000	2000	19	11	173
		s.c.	900–3000	2000	15	11	136
		i.c.	900–3000	2000	12	9	133
	Every 3 hr days 1, 5 and 9	i.v.	6·7–23	15	19(1)	10	190(1)
		s.c.	6·7–23	15	18	11	164
		i.c.	6·7–23	15	13	9	144

Tumour was implanted on day 0 and treatment was started 24 hr later (Day 1). There were 10 animals per dose level. Dose levels were separated by 0·18 log dose intervals.

The number of animals treated at the optimum dose who survived 50 days after the end of dosing are given in parentheses. Fifty day survivors have been ignored when calculating T/C.

The two more lipophilic AMSA agents (NSC150440 and NSC159862) significantly inhibited i.c. L1210 while the more polar agent NSC156303 failed in such tests.

When administered on an 8 × 3 hr schedule to animals with i.p. implanted L1210, NSC156303 provided greater life extensions than if given as a single dose (Table 2). However, when tumour had been implanted i.v. or s.c. there was no significant difference between the survival times recorded with the two different dosage schedules (Table 4). The differences in life extensions obtained with varying dosage schedules of cytosine arabinoside were also attenuated as tumour and drug administration site were increasingly separated (Tables 2, 4).

An investigation of the capacity of the two more lipophilic AMSA agents to eradicate varying sized inocula of L1210 cells showed that 3 doses at 4 day intervals would clear more than 50% of animals of an i.p. leukaemic inoculum of 10^4 cells (Table 5). No long term survivors resulted with the same dosage regimen if the identical number of tumour cells were implanted s.c. With larger i.p. inocula (10^5) few long term survivors resulted.

It was found that doubling the length of the treatment period (Table 5) would allow the eradication of 10^5 i.p. implanted leukaemic cells and even provide a percentage of survivors when the same number of cells were implanted s.c. With large tumour inocula (10^6 i.p. or s.c.) the more protracted treatment did not eradicate all L1210 cells, but there was a marked increase in survival times over those recorded with shorter dosing periods. The data demonstrates that protracted dosing with NSC159862 will provide tumour cell kills of > 99·999% of an i.p. implanted L1210 leukaemia population. The results obtained on protracted treatment also demonstrate the superiority of NSC159862 over NSC150440.

Effects on first generation transplants of C_3H/HeJ spontaneous mammary tumours

Mice with established, moderately large, first generation transplants of this mammary tumour were treated with the three AMSA agents using only one dosage schedule (daily 1–10). The tumour volumes recorded, during and after treatment, are plotted on a logarithmic scale against time in Fig. 3. Earlier research in this laboratory had shown that our positive control agent, cytosine arabinoside, at 15 mg/kg per dose every 3 hr on days 1, 5 and 9 did not significantly affect the growth of this tumour.

With the dosage schedule and doses used

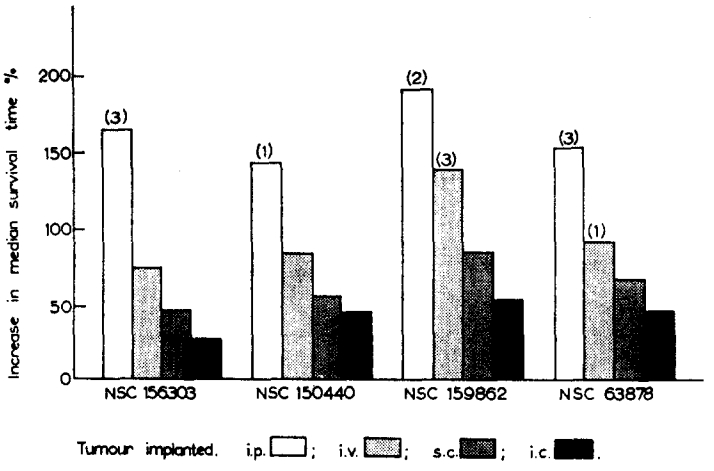


Fig. 2. Variation in life extensions with changing site of inoculation of 10^5 L1210 cells when agents were applied at their optimal dose and schedule.

Agent	*mg/kg per dose*	*Schedule*
*NSC*156303	4·4	*Every 3 hr day 1*
*NSC*150440	20	*Single, days 1, 5 and 9*
*NSC*159862	87	*Single, days 1, 5 and 9*
*NSC*63878	15	*Every 3 hr day 1, 5 and 9*

Numbers of animals surviving 50 days after the end of dosing are shown in parentheses at the top of the requisite column.

Table 5. Effect of more protracted dosage against varying sized inocula of L1210 leukaemia implanted either i.p. or s.c

Treatment schedule	Tumour site	Tumour inoculum	Median survival times		T/C%
			Treated	Control	
		*NSC*150440 *dosed intraperitoneally at 20 mg/kg per dose*			
Days 1, 5 and 9	i.p.	10^4	24(6)	12·5	192(6)
	i.p.	10^5	23(1)	9·5	242(1)
	i.p.	10^6	19	8	200
	s.c.	10^4	21	13	162
	s.c.	10^5	17	11	154
	s.c.	10^6	17	10	170
Days 1, 5, 9, 13, 17 and 21	i.p.	10^5	26(4)	10	260(4)
	i.p.	10^6	29(1)	8·5	342(1)
	s.c.	10^5	27	11	245
	s.c.	10^6	23	10·5	218
		*NSC*159862 *dosed intraperitoneally at 87 mg/kg per dose*			
Days 1, 5 and 9	i.p.	10^4	—(10)	12	—(10)
	i.p.	10^5	29(2)	10	290(2)
	i.p.	10^6	24	8	300
	s.c.	10^4	23	13	177
	s.c.	10^5	21	11·5	183
	s.c.	10^6	18	10	180
Days 1, 5, 9, 13, 17 and 21	i.p.	10^5	—(10)	10	—(10)
	i.p.	10^6	34(4)	8	425(5)
	s.c.	10^5	29(2)	11·5	252(2)
	s.c.	10^6	26	10	260

Tumour was implanted on day 0 and treatment was started 24 hr later (Day 1). There were 10 animals per dose level. Dose levels were separated by 0·18 log dose intervals.

The number of animals treated at the optimum dose who survived 50 days after the end of dosing are given in parentheses. Fifty day survivors have been ignored when calculating T/C.

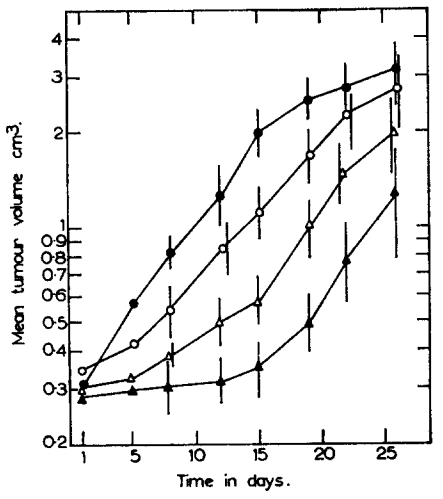


Fig. 3. Effect of the three AMSA variants on first generation transplants of C_3H/HeJ spontaneous mammary tumours.

Animals were dose i.p. daily, days 1–10. Doses used (mg/kg per day): NSC156303 (○) 4·4; NSC150440 (△) 4·9; NSC159862 (▲) 20. Doses 0·18 log interval higher and lower proved toxic and less active respectively. Controls (●) were injected with water alone. Standard errors of the mean tumour volumes are shown by vertical lines.

(Fig. 3) there was an excellent separation of the tumour inhibitory effects produced by the 3 AMSA agents. On day 15 the mean tumour volumes of all drug treated groups were significantly smaller ($P < 0·001$; Student's t-test) than that of the control groups. The differences in tumour volumes between the 3 drug treated groups were also significant ($P < 0·001$) at this time. The most polar agent (NSC156303) was least effective; NSC150440 was more effective and the most lipophilic congener (NSC159862) provided the best tumour growth inhibition. The difference in mean tumour volumes between the control groups and that treated with NSC156303 was less significant ($0·2 > P > 0·1$) on day 26. The mean tumour volumes of the groups treated with NSC150440 and 159862 were still significantly smaller than those of controls on day 26 ($P < 0·001$).

There was no observable regression of any of the tumours below their original volumes during or after drug treatment. The resistance of this virally induced tumour to conventional antitumour agents is well attested [17] and the growth inhibition produced by NSC159862 is of a high order.

Effects on the B16 melanocarcinoma

Representative members of the AMSA series have been evaluated by the American National Cancer Institute. We are indebted to Dr. John M. Vendetti, Drug Evaluation Branch DCT, NCI for the screening details presented (Table 6). NCI evaluation of all three AMSA compounds dealt with in this paper is not yet complete but the following results were obtained with NSC156303 against the B16 malignant melanoma test system. As observed with the L1210 leukaemia, there is a broad range of doses providing tumour inhibitory effects.

Table 6. Effects of NSC156303 against B16 malignant melanoma

Dose (mg/kg per dose)	Survival times (days)* Treated	Controls	T/C%
12·0	7·0	20·0	
6·0	41·0(1)	20·0	205(1)
3·0	42·5(3)	20·0	212(3)
1·5	32·5	20·0	162
0·80	39·0	22·0	177
0·40	31·0	22·0	140
0·20	26·0	22·0	118

Tumour brei was implanted i.p. day 0 in BDF_1 mice; animals were dosed i.p. daily from day 1 to 9. There were six animals per dose level. Survivors were evaluated on day 45.

*45-day survivors given in parentheses.

Effects against the P388 lymphocytic leukaemia

The following screening data was obtained by DCT, NCI utilizing an early sample of NSC156303 that had been supplied as the hydrochloride salt (NSC141549). The entries in Table 7 are representative of a large volume of

Table 7. NSC141549 vs P388 lymphocytic luekaemia

Dose (mg/kg per injection)	Survival times (days)* Treated	Controls	T/C%
12·5	7·0	11·0	
9·4	29·5(1)	11·0	268(1)
6·3	30·0(3)	11·0	272(3)
4·1	25·0	11·0	227
3·1	23·5	11·0	213
2·0	19·5	11·5	169
1·3	20·0	11·5	173
0·85	19·5	10·0	195
0·57	21·0	10·0	210
0·37	14·0	10·0	140
0·25	15·0	10·0	150
0·16	14·5	12·0	120
0·11	15·0	12·0	125

10^6 leukaemic cells were implanted i.p. in BDF_1 mice on day 0; animals were dosed i.p. daily from day 1 to 9. There were 6 animals per dose level. Survivors were evaluated on day 30.

*30-day survivors in parentheses.

screening data obtained in the P388 leukaemia system. Again, the range of doses evincing significant tumour inhibition is noteworthy.

DISCUSSION

To be capable of effecting radical cure an antitumour agent must be able to reach all tumour cells in lethal concentration [3]. The ability of the AMSA agents to effectively reach remote tumour cells is shown by the life extensions obtained when the drugs were administered to animals implanted with L1210 at various sites (Tables 2, 4, 5). There was a clear discrimination against the most polar agent (NSC156303); no significant life extension being observed in any test with this drug against i.c. inoculated leukaemia (Table 4). The congener differing only in the presence of a further methyl group (NSC150440) could significantly affect i.c. implanted L1210. The most lipophilic agent examined (NSC159862) was marginally more effective than the monomethyl congener against i.c. L1210 but showed clear superiority when the 2 drugs were compared using protracted dosage schedules (Table 5). The agents can accordingly be ranked in decreasing order of their ability to effect remote tumour depots; NSC159862; NSC150440; NSC156303.

Rall's studies [18] demonstrate that rate of penetration of the blood–brain barrier by basic drugs is proportional to the amount and the lipophilic character of neutral drug species present at physiologic pH's. The pK_a and partition coefficients provided in Table 1 are then in agreement with the observed order of activity seen in tests with the 3 AMSA variants against i.c. implanted leukaemia.

From the life extensions observed in i.p. L1210 tests alone (Table 2) it would be difficult to discriminate between the 3 agents. The ranking method used relies heavily on the results of tests against remote tumour implants. It is not possible to decide, from screening data alone, if there are intrinsic differences in the lethality of the agents towards leukaemic cells. The differences in life extensions recorded may merely reflect changing pharmaco-kinetic properties; the better distributing agents being able to effectively reach a greater proportion of a given tumour population.

The most polar agent, NSC156303, appeared schedule dependent when administered i.p. to animals with i.p. L1210 (Table 2). Rate of drug movement to a site of action is in most cases controlled by partition dependent processes [4]; an optimum lihophilic character being necessary for a drug to be able to adequately blockade a distant site. As a corollary, rate of drug movement from the site of drug administration should also be partition controlled. More polar drugs could be expected to have greater residence times at the site of administration. The schedule dependency seen with NSC156303 against i.p. L1210 may then be due to pharmacokinetic properties resulting from the more polar nature of this agent. There was no significant evidence of schedule dependency against either s.c. or i.c. implanted L1210, with any of the AMSA agents examined (Table 4).

Against i.p. or i.v. L1210 cytosine arabinoside showed clear evidence of schedule dependency (Tables 2, 4). However, such dependency was less apparent against s.c. or i.c. implanted leukaemia (Table 4). Pharmacokinetic properties can also be expected to play a role in the tumour inhibitions obtained with this drug. The tumour cell kill produced in any body compartment by a cycle specific agent [19] will depend on the time for which tumour effective drug concentrations persist [15] and the growth fraction of the target cells [20]. The concentration of a drug pervading any body compartment will be determined by pharmaco-kinetic parameters. The anatomic location of a tumour population may then determine whether significant evidence for schedule dependency will be attainable.

The variant (NSC159862) showing greatest activity against transplants of C_3H spontaneous mammary tumours (Fig. 3) is that agent which provided greatest life extension in tests against i.c. L1210 (Table 4). If the assumption is made that greater activity against i.c. leukaemia results from better distribution of an agent then it may be suggested that the varying inhibitions of the C_3H tumour seen (Fig. 3) also reflect the different distributive capacities of the agents.

Members of the AMSA series have been shown to inhibit the growth of the L1210 and P388 leukaemias, the B16 melanocarcinoma and first generation transplants of the spontaneous C_3H mammary adenocarcinoma. These tumours vary in growth habit (ascitic or solid); growth fraction; cell cycle times and the length of intermitotic phases [20, 21]. A more detailed examination of the spectrum of action of these agents is clearly warranted. The reason for the selectivity towards tumour shown by these agents is under active investigation.

At hand we have more than 120 L1210 acitve AMSA variants. The results described suggest that further winnowing of this group might be achieved by testing each variant in turn against s.c. and i.c. implanted L1210. For further selection the capacity of agents to eradicate

10^6 s.c. implanted L1210 using protracted dosage schedules might be examined (cf. Table 5).

The structure activity relationships with all the AMSA variants presently available suggest that the cation is the biologically important species of these acridine derivatives. In agreement NSC159862, having a reduced pK (Table 1), provides less of the biologically active cation and is consequently less molar potent; the optimum doses required with this agent are 4·1–6·5 times greater than those of the other 2 variants examined. It appears that delicate control of pK will be necessary to ensure that future AMSA congeners have desirable distributive properties while retaining acceptable molar potency.

The formulae of the AMSA agents, as presented in two dimensional form (Fig. 1), suggest there is overall molecular planarity. Molecular models demonstrated that marked steric interaction between the 1 and 8 hydrogen atoms of the acridine system and the 9-methanesulphonanilide component could be expected. An X-ray crystallographic analysis of the lead molecule (4′-[(9-acridinyl)amino] methanesulphonanilide) [22] has shown that, in the crystal, the C_9-N-$C_{4'}$ bond angle is widened from usually observed values. In addition, the 9-methanesulphonanilide component is rotated so that the ring plane lies at right angles to the plane of the acridine ring system.

It is not surprising to find that the AMSA agents, as acridine derivatives, bind strongly to DNA *in vitro* [23]. As with the 9-aminoacridine there are two modes of binding observed [24] suggesting that, despite the angular 9-methanesulphonanilide ring, the acridine portion of the AMSA agents can still intercalate between adjacent base pairs of DNA duohelices. The observed binding constants of the AMSA agents to DNA are greater than that measured for 9-aminoacridine under the same conditions [23].

Of the 3 agents discussed the first submitted (NSC141549; the hydrochloride salt corresponding to NSC156303) has been selected by D.R.D. Chemotherapy, N.C.I. as a candidate for clinical trial.

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REFERENCES

1. B. F. Cain, G. J. Atwell and R. N. Seelye, Potential antitumour agents. 10 Bisquaternary salts. *J. med. Chem.* **12,** 199 (1969).
2. G. J. Atwell, B. F. Cain and R. N. Seelye, Potential antitumour agents. 12. 9-Anilinoacridines. *J. med. Chem.* **15,** 611 (1972).
3. H. E. Skipper, F. M. Schabel, jr., W. S. Wilcox, On the criteria and kinetics associated with curability of experimental leukaemia, *Cancer Chemother. Rep.* **35,** 35 (1964).
4. M. S. Tute in *Advances in Drug Research*, Vol. 6, p. 6. (Edited by N. J. Harper and A. B. Simmonds). Academic Press, London and New York (1971).
5. A. Albert and E. P. Serjeant, *The Determination of Ionization Constants*, Chapman & Hall, London (1971).
6. A. Leo, C. Hansch and D. Elkins, Partition coefficients and their uses. *Chem. Rev.* **71,** 525 (1971).
7. J. Gelzer and P. Loustalot, Biological and chemotherapeutic studies on primary mammary tumours in C_3H and C_3H_0 mice. *Int. J. Cancer* **2,** 179 (1967).
8. A. Albert, *The Acridines*, p. 437. 2nd Edn. (1966). Edward Arnold, London.
9. C. Hansch, A. Leo and D. Nikaitani, On the additive—substituent character of partition coefficients. *J. org. Chem.* **37,** 3090 (1972).
10. P. N. Craig, Interpedendence between physical parameters and selection of substituent groups for correlation studies. *J. med. Chem.* **14,** 680 (1971).
11. J. G. Topliss and M. D. Yudis, π-Substituent constants for the 2H-1,2,4,-benzothiadiazine-1,1-dioxide system. *J. med. Chem.* **15,** 400 (1972).
12. J. M. Venditti, Treatment schedule dependency of experimentally active antileukaemic (L1210) drugs. *Cancer Chemother. Rep.* **2** (**3**), 35 (1971).
13. H. E. Skipper and L. H. Schmidt, A manual of quantitative drug evaluation in experimental tumour systems. *Cancer Chemother. Rep.* **17,** 1 (1962).
14. J. M. Venditti, M. C. Barratta, N. H. Greenberg, B. J. Abbott and I. Kline, Studies of the L1210 antileukaemic activity of O-2,2′-cyclocytidine monoacetate—comparison with cytosine arabinoside. *Cancer Chemother. Rep.* **56** (**1**), 483 (1972).

15. H. E. SKIPPER, F. M. SCHABEL, JR. and W. S. WILCOX, Experimental evaluation of potential anticancer agents—XXI. Scheduling of arabinosylcytosine to take advantage of its *S*-phase specificity against leukaemia cells. *Cancer Chemother. Rep.* **51,** 121 (1967).
16. J. E. SHACKLEY, A computer model for tumour growth and chemotherapy and its application to L1210 leukaemia treated with cytosine arabinoside (NSC63878). *Cancer Chemother. Rep.* **54** (**1**), 399 (1970).
17. J. SANDBERG and A. GOLDIN, Use of first generation transplants of a slow growing tumour for evaluation of new cancer chemotherapeutic agents. *Cancer Chemother. Rep.* **55,** 233 (1971).
18. D. P. RALL, J. R. STABENAU and C. B. ZUBROD, Distribution of drugs between blood and cerebrospinal fluid. *J. Pharm. exp. Ther.* **125,** 185 (1959).
19. H. E. SKIPPER, F. M. SCHABEL, JR., BRUCE MELLETT, J. A. MONTGOMERY, L. J. WILKOFF, H. H. LLOYD, R. WALLACE BROCKMAN, Implications of biochemical, cytokinetic, pharmacologic and toxicologic relationships in the design of optimal therapeutic schedules. *Cancer Chemother. Rep.* **54** (**1**), 431 (1970).
20. L. SIMPSON-HERREN and H. H. LLOYD, Kinetic parameters and growth curves for experimental tumour systems. *Cancer Chemother. Rep.* **54** (**1**), 143 (1970).
21. D. P. GRISWOLD, JR., Consideration of the subcutaneously implanted B16 melanoma as a screening model for potential anticancer agents. *Cancer Chemother. Rep.* **3** (**2**), 315 (1972).
22. D. A. SWANN, PH.D., thesis, University of Auckland, New Zealand (1971).
23. W. R. WILSON, B. F. CAIN and B. C. BAGULEY, Manuscript in preparation.
24. L. S. LERMAN, Structural consideration in the interaction of DNA and acridines. *J. mol. Biol.*, **3,** 18 (1961).

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Letter to the Editor

Properties of the Mouse Cells Transformed with DNA Containing the Murine Sarcoma Provirus

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RECENTLY, we reported the recovery of the murine sarcoma virus (MSV) genome after the infection of mouse cells with nuclear DNA from MSV-transformed non-virus producing cells [1]. In our initial studies the biological activity of the nuclear DNA was demonstrated by its ability to transform normal mouse cells *in vitro*. The MSV genome could then be "rescued" from the transformed cells with a helper Rauscher leukaemia virus (RLV), and the pseudotype virus produced (MSV-RLV) transformed mouse cells *in vitro*.

In this paper we wish to report the properties of 3T3-BALB/c cells which were transformed by the MSV-DNA provirus but did not contain a rescuable MSV. The properties of the transformed cells which are outlined in this paper can be summarised as follows: (*a*) pleomorphic cytology, (*b*) an abnormal karyotype containing large marker metacentric chromosomes not present in the normal mouse karyotype and (*c*) highly malignant properties as evidenced by the induction of a rapidly developing sarcoma in the inoculated BALB/c mice. These three inter-related properties suggest that the cells have been transformed by the MSV-DNA provirus, in spite of the fact that a biologically rescuable virus could not be demonstrated.

DNA was extracted from MSV-transformed but non-virus producing mouse cells, the D-243 line [2], by a similar procedure to the one we outlined earlier [1], 3T3-BALB/c cells were seeded into four 50 mm plastic plates at a concentration of 3×10^5 cells per plate. On the following day, each plate was inoculated with 5 ml of Dulbecco's modified Eagle's medium (DMM) containing 20 μg of diethyl-aminoethyl-dextran (DEAE-D) per ml. After 30 min of incubation, the cells were washed twice with DMM and to one set of plates 40 μg of the D-243 DNA was added. Another set of plates were used as controls. The DNA containing plates were incubated at 37°C for one hour before 10% calf serum in DMM was added. Both DNA infected and non-infected 3T3-BALB/c cultures had been grown for five months before this study was conducted. During this period, both the infected and non-infected cells were trypsinized and subcultured ten times (at an average of two-week intervals). Both DNA-treated and untreated cultures grew during this period as stationary cultures. May–Grünwald–Giemsa stained cultures revealed that the control cells had a single nucleus of uniform size and shape (Fig. 1), while the DNA-treated cultures contained a pleomorphic cell population of variable size and shape. Many of them were greatly enlarged, some of them contained a very large nucleus and most of the large cells were multinucleated. The nuclei in these cells were of abnormal size and shape. In some cells nuclear fragments were scattered through the cytoplasm (Fig. 2).

For karyotype analysis a procedure which

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was described earlier [3] was used. Nineteen chromosome spreads of the control cultures were examined and all were found to have normal karyotypes. On the other hand, of the 20 chromosome spreads which were analysed from the DNA-infected cells, 8 (40%) had an abnormal karyotype which contained large marker metacentric chromosomes while the other 12 had normal chromosomes. Such metacentric chromosomes (Fig. 3) were not present in the normal mouse karyotype.

The oncogenic properties of the DNA-transformed cells were tested by the inoculation of eight weeks old BALB/c mice with the cultured cells. Ten mice were inoculated with the transformed cells, while 5 mice were inoculated with the control cells. Each animal was injected sub-cutaneously in the thigh with a volume of 0·2 ml containing 5×10^5 cells of the corresponding cultures. All 10 mice inoculated with the transformed cells developed tumours. In 2 mice tumours appeared at the injection site in three weeks: the other 8 mice also developed large tumours at the site of injection within four weeks of inoculation. The tumour increased quickly in size and the animals were killed on day 26 when most were moribund. Post-morten examination of the mice revealed a very large subcutaneous soft tissue growth and masses of similar tissue in the abdominal cavity. Histological examination of the tumour tissue revealed that it was an undifferentiated sarcoma. Blood smears from mice that developed the sarcoma and stained with Leishman did not contain leukaemic cells. The five mice which were inoculated with the control cells developed no signs of tumours during the three months of observation.

The tumours that developed in the 10 mice were collected separately from each animal. A 10% suspension was prepared from each tumour by homogenization of the tissue in DMM. The suspensions were then spun at 2000 rev/min for 15 min, and the supernatant was passed through a 0·45 μm Millipore filter. Both filtrates from homogenized tumour cells and filtrates of the cultured DNA-transformed cells were assayed for infectious MSV using the focus formation assay. In addition, filtered culture fluid from an MSV rescue experiment was assayed.

For the assay of all the above tumour and cultured cell filtrates, 3×10^5 normal 3T3-BALB/c cells were seeded on 50 mm plastic plates. On the following day each plate was treated for 30 min with 100 μg of DEAE-D and then infected separately with the different cell filtrates. The infected plates were changed every two days and were checked for the development of foci of transformation for two weeks. In none of those plates could a focus of transformed cells be detected. Only cultured fluid from the rescue experiments in which HT-1 cells were used as a known source of MSV genome caused the appearance of foci of transformation (Table 1). The MSV rescue experiment was performed with two mixed cell populations. One cell mixture was made of 5×10^5 DNA transformed 3T3-BALB/c cells, 3×10^5 RLV infected 3T3-BALB/c cells and 10^6 normal 3T3-BALB/c cells. The second cell mixture was used as a control for the MSV "rescue" procedure and was done with the HT-1 cells (5×10^4) as a known source of MSV genome, using similar cell concentrations of RLV infected and normal 3T3-BALB/c cells. No biologically active MSV could be demonstrated in the DNA-transformed mouse cells or in any of the tumour cells. The failure to recover the MSV genome from the DNA transformed cells using RLV as a helper virus indicates that if murine sarcoma viral information was integrated into the cells following the DNA infection, only an incomplete provirus was integrated.

Hill and Hillova [4] were first to demonstrate that cellular DNA extracted from Rous sarcoma virus (RSV)-transformed rat XC cells, contains the genetic information of this virus. We have recently shown that nuclear DNA from MSV transformed non-virus producing mouse and hamster cells contains the mouse sarcoma provirus. In our initial studies we reported the successful recovery of the MSV genome from the DNA-infected mouse cells.

Table 1. In vitro *assay for infectious MSV*

Filtrates (0·45 μ) used for the inoculation of 3T3-BALB/c cells	Appearance of foci of transformation
Tumour tissue of mice 1–10	—
D-243-DNA transformed 3T3-BALB/c cells	—
D-243-DNA transformed 3T3-BALB/c cells + RLV-3T3 cells + 3T3-BALB/c cells	—
HT-1 cells + RLV-3T3 cells + 3T3-BALB/c cells	+

No infectious MSV could be isolated from any of the 10% tumour suspension.
Likewise DNA-transformed 3T3 cells did not release an infectious MSV even in the rescue experiments in which RLV was used as a helper virus. However the MSV genome could be rescued from the HT-1 cells.

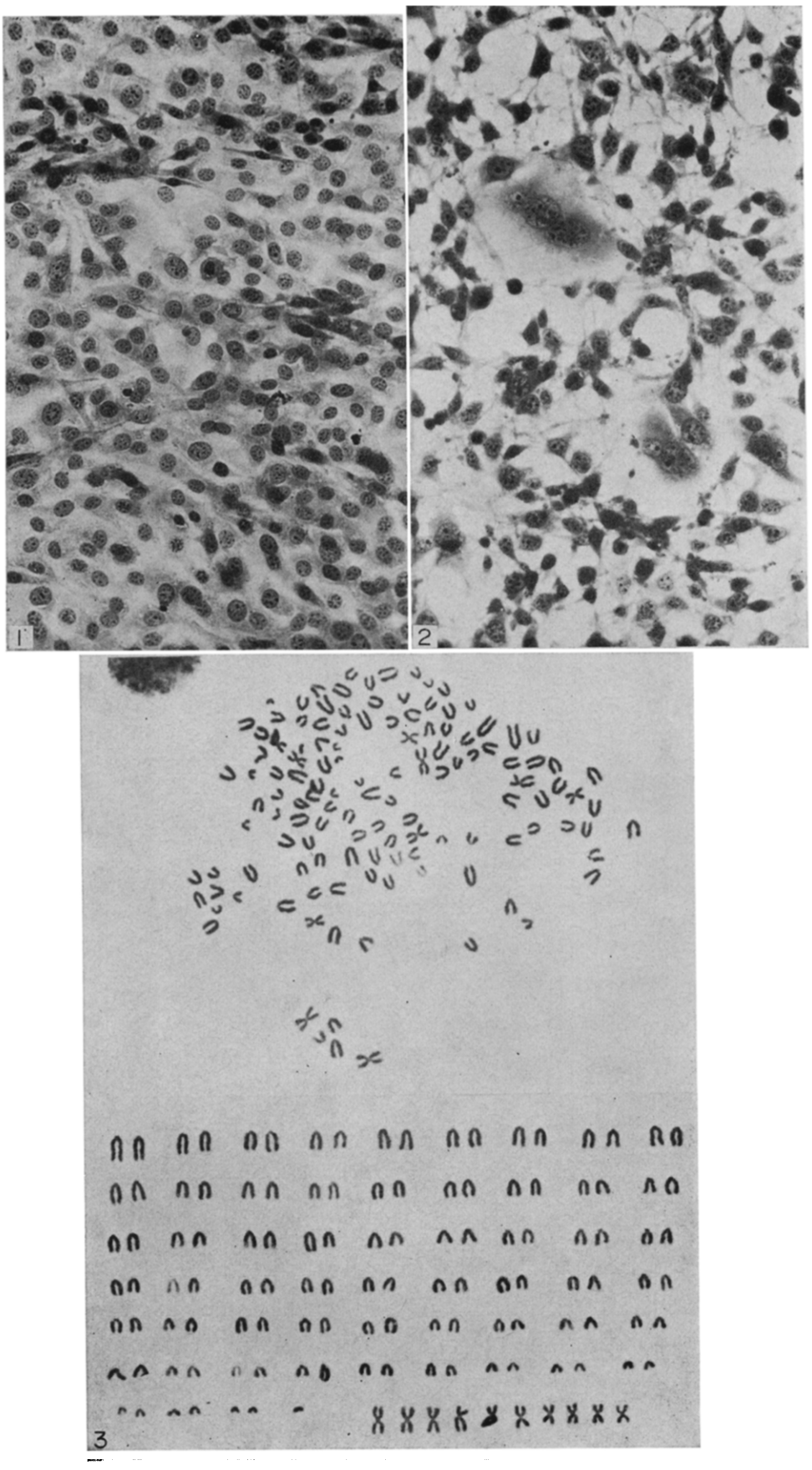


Fig. 1. *Uninfected 3T3–BALB/c cultures. Uniform population of mononuclear cells is clearly demonstrated.*

Fig. 2. *D–243–DNA infected 3T3–BALB/c culture. This figure demonstrates the morphological heterogeneity not seen in the mononuclear cells of this control culture. Two typical multinucleated giant cells are also present.*

Fig. 3. *Karyotype of 3T3–BALB/c cells transformed by MSV–DNA. This cell contains a total of* 125 *chromosomes which includes ten large metacentric "marker" chromosomes normally not present in mouse karyotype. The normal mouse karyotype contains* 44 *acrocentric chromosomes.*

(to face p. 552)

In this paper we describe the properties of mouse cells transformed by infection with a similar DNA preparation but in which an incomplete murine sarcoma provirus was integrated. The cytological abnormalities induced in the DNA-treated cells resemble the cytological abnormalities found in hamster [3] and rat cell lines carrying latent RSV and MSV genomes [5]. Likewise, the cytogenetic changes, namely the appearance of large marker metacentric chromosomes which are not present in normal mouse karyotypes, correspond to similar abnormal chromosomes in the D-243 mouse cells (unpublished observation) and the transformed hamster and rat cells studied [3, 5]. In addition, the DNA-treated cells were found to be highly oncogenic when inoculated into adult mice. The failure to rescue and demonstrate the presence of the MSV genome in the transformed cells suggests that an incomplete murine sarcoma provirus was integrated into the cell genome, sufficient genetic information being incorporated to cause cellular transformation. This situation may be analogous with some of the naturally occurring spontaneous malignant transformations in man in which no virus information can be rescued or demonstrated by the available techniques, but in which an incomplete viral genome may nevertheless be present.

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REFERENCES

1. A. Karpas and C. Milstein, Recovery of the genome of murine sarcoma virus (MSV) after injection of cells with nucleus DNA from MSV transformed non virus producing cells. *Europ. J. Cancer* **9,** 295 (1973).
2. R. H. Bassin, L. A. Phillips, M. J. Kramer, D. K. Haapalen, P. T. Peebles, S. Nomura and P. J. Fischinger, Transformation of mouse 3T3 cells by murine sarcoma virus: release of virus-like particles in the absence of replication murine leukaemia helper virus. *Proc. nat. Acad. Sci.* **68,** 1520–1524 (1971).
3. A. Karpas, J. Cawley, E. Tuckerman, R. Flemens and F. G. Hayhoe, Cytochemistry, cytogenetics and ultrastructure of hamster tumour cells carrying mouse sarcoma viral genome (HT–1 cells). *Brit. J. Cancer* **25,** 770 (1971).
4. M. Hill and J. Hillova, Virus recovery in chicken cells tested with rous sarcoma cell DNA. *Nature New Biol.* **237,** 35 (1972).
5. A. Karpas, J. Cawley and E. Tuckerman, Cytological, cytogenetic and ultrastructural studies of rat cell lines carrying avian and murine sarcoma genomes. *Z. Krebsforch* **78,** 51 (1972).

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HL–A Antigens and Breast Cancer

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Abstract—*HL–A antigens were determined in 200 breast cancer patients and 200 matched controls. No significant differences were found between the frequencies of HL–A antigens in the two groups. Also, when the patients were divided according to several clinical criteria, no significant associations with any of the HL–A antigens were observed.*

INTRODUCTION

SEVERAL observations indicate that hereditary factors may be of importance in breast cancer. There is a three-fold increased frequency of the disease in first degree relatives of patients suffering from breast cancer. Breast cancer may also concentrate within certain families. These observations, however, could not only be explained by the action of one or several genes, but could also be due to environmental factors. Studies of the association between breast cancer and several blood group systems have so far given inconclusive results [1–4].

In inbred strains of mice, there is a relationship between histo-compatibility genes and the susceptibility to several RNA-tumor viruses. This was first reported by Lilly [5] for the Gross leukemia virus and later by Muhlbock [6] for certain types of mammary tumor virus (MTV).

In recent years, much evidence has accumulated to prove the existence of a human MTV [7].

These observations prompted us to investigate the relationship between HL–A antigens and breast cancer. Frequencies of HL–A antigens in patients with breast cancer were compared to those in matched controls.

PATIENTS AND METHODS

Patients

From November, 1971, till November, 1972, two hundred Dutch patients with breast cancer were studied. All patients were from the outpatient department of the Netherlands Cancer Institute. The majority had been operated upon after 1965. Full clinical records and details of the operative procedures, tumor histology and course of the disease, were available. Special care was taken to obtain an accurate family history with regard to breast cancer. When metastases were present we noted the symptom-free interval after operation and the localization of metastases. Three types of metastases were distinguished according to localization: (a) local and skin metastases, 33 cases; (b) osseous metastases, 40 cases; (c) visceral metastases, 24 cases. When there was localization in more than one system, the patient was classified in the group of the worst prognosis (Cooperative Breast Cancer Group, Progress Report, 1961). The median age of the patients was 51 years. The nulliparous rate was 32 per cent. Of the 200 patients, 108 were post-menopausal, 32 had undergone castration and 18 had an artificial menopause induced for gynaecological reasons. Only 42 patients were premenopausal. The histology was divided in three groups: (a) undifferentiated carcinoma, 101 cases; (b) adenocarcinoma, 81 cases; and (c) miscellaneous group of 18 cases, in which were comprised medulary carcinoma, intraductal carcinoma, Paget's disease and mucoid carcinoma. In 97 patients the axillary nodes were negative at histological examination, in 100 patients positive axillary nodes were found and in 3 patients we had insufficient information. Bilateral breast tumors were found in 11 cases. One hundred and three patients were

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free from metastases at the time of investigation. In a quarter of these patients the interval after mastectomy and the time of investigation was less than 24 months. In patients with metastases the symptom-free period was shorter than 24 months in half of the patients.

First degree relatives with breast cancer were found in the history of 39 patients. Second degree relatives with breast cancer were found in another 42 patients.

Controls

The controls were healthy unrelated Dutch women matched for parity, age by decades, and for habitat. The last selection was done to decrease the possible influence of environmental factors. The median age was 54 years. The family history for breast cancer was positive in 28 control women. In only 7 cases the tumor occurred in first degree relatives.

Methods

The histo-compatibility testing was carried out by the Department of Immunohaematology of the University of Leiden. The HL–A typing was initially performed with the Kissmeyer–Nielsen microlymphocytotoxic technique and later with the standard N.I.H. microlymphocytotoxic technique. In most of the cases the leukocytes were also tested in the microagglutination test. Well defined antisera were used to detect the HL–A antigens. Correlations between antigen frequencies and clinical data were calculated by computer analysis, and expressed as χ^2.

RESULTS

The HL–A types of patients and controls are set out in Table 1. Only χ^2 values under 2 were found, indicating that there are no significant differences between patients and controls. When this material was analysed for the results of the agglutination test and the supertypic groups, such as 4^a and 4^b, again no significant differences were found.

To exclude the possibility that significant association of certain HL–A antigens with part of the patients was diluted out by imput of a population which did not show this association, the material was divided with respect to several clinical parameters. These included: age, parity, familial occurrence of breast cancer, bilateral cancer, axillary node involvement, type of spread, symptom-free interval after surgery and histological tumor type. None of these parameters showed a significant correlation with the frequency of any of the HL–A antigens. On further analysis we noticed that the combination of HL–A 9 and HL–A 27 appeared to be more frequent in patients than in the controls. The frequency of this antigen combination was than compared to that of larger control group, composed of the 200 patient's controls and 1272 unrelated persons from the Leiden panel. The increased frequency of the combination HL–A 9 and HL–A 27 was highly significant (Table 2). But after correction for the 135 possible antigen combinations, a P value of $0{\cdot}135 \leqq P \leqq 0{\cdot}675$ was

Table 1. *Comparison of antigen frequency in patients and controls*

HL–A antigen	Patients (200)	Controls (200)	χ^2
1st segregant series			
HL–A 1	71	71	0·000
HL–A 2	109	107	0·039
HL–A 3	55	60	0·250
HL–A 9	41	31	0·999
HL–A10	13	9	0·159
HL–A11	26	19	0·489
HL–A28	14	26	1·467
W19	21	21	0·014
2nd segregant series			
HL–A 5	28	25	0·089
HL–A 7	50	59	0·809
HL–A 8	59	51	0·639
HL–A12	34	45	1·210
HL–A13	13	8	0·250
HL–A14	10	5	0·268
HL–A17	13	20	0·489
HL–A27	18	12	0·359
W 5	35	26	0·848
W10	30	42	1·222
W15	45	36	0·809
W18	9	6	0·248
W21	4	5	0·010
W22	8	8	0·000

Table 2. *Combination of HL–A* 9 *and HL–A* 27 *in patients and controls*

HL–A Antigen		Patients	Controls	Total
HL–A 9 HL–A27	+	9	17	26
HL–A 9 HL–A27	—	191	1455	1646
		200	1472	1672

$\chi^2 = 12{\cdot}9$.
$P = 0{\cdot}003$ uncorrected. $= 0.0003$
Yates correction $P = 0{\cdot}001 \leqq P \leqq 0{\cdot}005$.

estimated, which was below the level of significance.

Family studies were undertaken to establish whether these two antigens were carried on one chromosome and thus constituted a haplotype. In 8 of the 9 patients we were able to investigate one or several family members. In 5 out of 8 patients the combination constituted a haplotype. In the other three cases the haplotypes were HL-A 2–HL-A 27 twice, and HL-A 30–31—HL-A 27 once.

DISCUSSION

Associations between HL-A antigens and several diseases in man have been reported. In gluten sensitive enteropathy and myasthenia gravis, the association with HL-A 8 was highly significant and there was also a significant association of ankylosing spondylitis with HL-A 27 [8–10]. The study of such associations in malignant diseases have sofar been unrewarding. Positive associations have been reported for acute lymphocytic leukemia and for Hodgkin's disease [11, 12]. When the studies were repeated, the associations could not always be reproduced. It has been suggested that some of the discordance in results could be attributed to serological problems, others to geographic differences in HL-A frequencies. For instance there may be an association of a disease with the product of a genetic locus which is in lonkage disequilibrium with the locus coding for a serologically defined HL-A antigen. This linkage disequilibrium may exist in some populations, but not in others [13].

Until now results of three studies of HL-A antigens and breast cancer have been reported. In the studies of Marsz and Benacerraf [14] and those of Cordon and James [15] no significant correlations were found. Patel and co-workers [16] found a significant excess of HL-A 7 in 52 patients with breast cancer, when compared to 123 healthy unrelated blood donors. These controls were not matched for age, sex or country of origin. According to the joint report of the 5th histocompatibility workshop, frequencies of HL-A 7 in the Caucasian population have a range of 2–39 [17]. The study of Patel and co-workers also illustrates the problem of comparing the frequencies of many antigens with one disease. It has been suggested that the statistical significance of an association may be overestimated because of first order error [18, 19]. For instance, one out of 20 comparisons taken at random can be expected to be significant at the 5% level. Therefore, the normal significance level should be multiplied by the number of comparisons made.

The results of our studies also fail to show a correlation between HL-A antigens and breast cancer. The increased frequency of the combination HL-A 9—HL-A 27 warrants further investigation. This kind of investigation is not sufficient to exclude any relation between HL-A types and breast cancer. In mice the correlation of increased susceptibility to RNA-tumor virus and H2-antigens could only be demonstrated in inbred strains.

In the search for linkage of genetic markers with breast cancer in man, the most fruitful approach may be the study of breast cancer families. Such studies would also allow the determination of HL-A haplotypes. A number of these families are now under investigation.

REFERENCES

1. P. Speiser, Krankheiten und blutgruppen. *Wien. klin. Wschr.* **70,** 315 (1958).
2. I. S. Goldenberg and M. A. Hayes, Breast carcinoma and ABO blood-groups. *Cancer* **11,** 973 (1958).
3. Relation between breast cancer and S blood-antigen system. Report from Boston collaborative drug surveillance programma. *Lancet* **i,** 301 (1971).
4. P. Morosini, E. G. Lee, M. N. Jones and M. P. Vessey, Breast cancer and the S blood-group system. *Lancet* **i,** 411 (1972).
5. F. Lilly, The role of genetics in Gross virus leukemogenesis. Comparative leukemia research 1969. Bibl. haemat., No. 36 (Edited by R. M. Dutcher) pp. 213–220 Karger, Basel (1970).
6. O. Mühlbock and Anna Dux, Histocompatibility genes and mammary cancer in mice. Proc. symp. immunogenetics of the H-2 system, Liblice, Prague 1970, 123–128 Karger, Basel (1971).
7. J. Schlom, D. Colcher, S. Spiegelman, S. Gillespie, D. Gillespie, Quantitation of RNA tumorviruses and viruslike particles in human milk by hybridization to polyadenylic acid sequences. *Science (N.Y.)* **179,** 696–698 (1973).
8. P. L. Stokes, P. Asquith, G. K. T. Holmes, P. Mackintosh and W. T. Cooke, Histocompatibility antigens associated with adult coeliac disease. *Lancet* **ii,** 7769, 162 (1972).

9. C. P. ENGELFRIET, Personal communications (1972).
10. D. A. BREWERTON, MAEVE CAFFREY, F. D. HART, D. C. O. JAMES, ANNE NICHOLLS and R. D. STURROCK, Ankylosing spondylitis and HL-A 27. *Lancet* **i,** 903 (1973).
11. J. L. AMIEL, Study of the leucocyte phenotypes in Hodgkin's disease. Histocompatibility testing 79 Munksgaard, Copenhagen (1967).
12. E. THORSBY, A. BRATLIE and S. O. LIE, HL-A genotypes of children with acute leukemia: a family study. *Scand. J. Haemat.* **6,** 409 (1969).
13. J. J. VAN ROOD, Histocompatibility antigen systems and the control of the immune response: their relevance in disease susceptibility. *Neth. J. Med.* **16,** (1973).
14. E. MARTZ and B. BENACERRAF, Lack of association between carcinoma of the breast and HL-A specificities. *Tissue Antigens* **3,** 30 (1973).
15. ANNA L. CORDON and D. C. O. JAMES, HL-A and carcinoma of the breast. *Lancet* **ii,** 565 (1973).
16. R. PATEL, M. B. HABAL, R. E. WILSON, A. G. BIRTCH and F. D. MOORE, Histocompatibility (HL-A) antigens and cancer of the breast. *Amer. J. Surgery* **124,** 31 (1972).
17. Joint report of the Fifth International Histocompatibility Workshop. Histocompatibility testing 651 Munksgaard, Copenhagen (1972).
18. A. S. WIENER, Controversy in human genetics, blood groups and disease. *Amer. J. hum. Gen.* **22,** 476 (1970).
19. H. O. MACDEVITT and W. F. BODNER, Histocompatibility antigens, immune responsiveness and susceptibility to disease. *Amer. J. Med.* **52,** 1 (1972).

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The Ultrastructural Demonstration of Virus-Like Particles in Human Leukaemic Cells*

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Abstract—*This ultrastructural study is concerned with the description of virus-like particles in the cytoplasm of primitive human leukaemic cells. Such virus-like particles are shown not to be confined to a particular type of leukaemia, being seen in acute granulocytic leukaemia, acute lymphocytic leukaemia, and blastic crisis of chronic myeloid leukaemia. The morphology and distribution of the particles is considered in relation both to previous ultrastructural studies of possible virus particles in human leukaemias, and to the possible association between oncogenic viruses and this group of malignancies. Since C-type viruses have been shown to be the causative agent in an increasing number of animal leukaemias, the possibility that the virus-like particles described in this paper represent incomplete C-type virus is discussed.*

INTRODUCTION

THE ULTRASTRUCTURAL demonstration of characteristic C-type virus particles in avian and murine leukaemia [1] stimulated a search for similar particles in human leukaemia and lymphoma material. Many such ultrastructural studies involved the examination of pellets obtained by ultracentrifugation of leukaemic blood plasma [2–5]. However, the identification of virus-like particles among a mass of debris in plasma pellets is very difficult, and the results of such studies must therefore be viewed with considerable caution [6, 7]. Although the interpretation of electron microscope studies of ultra-thin sections of whole cells is more reliable than the examination of plasma pellets, the results of studies of thin sections of leukaemic cells have been conflicting [6]. Thus, a number of essentially negative reports have been made [8–11], together with a number of more or less convincing positive studies of which the reports of Dmochowski *et al.* [12, 13] and Seman and Seman [14] are of particular interest. These conflicting results encouraged us to report the occurrence in various human leukaemias of cytoplasmic virus-like particles resembling those described by Seman and Seman [14]. The differences between these virus-like particles and those of Seman and Seman are discussed. The possible significance of these virus-like particles is also considered.

MATERIAL AND METHODS

Eight cases of acute granulocytic leukaemia (AGL), 10 of acute myelomonocytic leukaemia (AML), 3 of erythro-leukaemia, 8 of acute lymphocytic leukaemia (ALL), 11 of chronic myeloid leukaemia (CML) including 2 of blastic crisis, and 5 of chronic lymphocytic leukaemia (CLL) were studied. The cases of acute leukaemia were classified according to the cytological and cytochemical criteria defined by Hayhoe *et al.* [15], and a Philadelphia chromosome was demonstrated in all the cases of CML and blastic crisis. The peripheral blood leucocytes were examined in all cases, and bone marrow leucocytes were also examined in the majority of cases. All material was

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fixed on the ward before further processing in the laboratory. Fixation was carried out for 1 hr at room temperature in 1·5% or 3% glutaraldehyde in 0·1M cacodylate–HCl (pH 7·4), and subsequent processing for electron microscopy was performed as previously described [16]. Thin sections were stained with uranyl acetate and lead citrate.

In those cases in which the virus-like particles were seen, freshly collected cells were fixed and then incubated in the cytochemical media of Graham and Karnovsky [17] and Novikoff and Goldfischer [18] before further processing in the manner described above.

Peripheral blood and bone marrow leucocytes from more than 30 normal individuals, which had been processed in an identical fashion as part of another study, were examined as control material.

RESULTS

Intracytoplasmic structures resembling virus particles (= virus-like particles) were seen in 5 separate cases of leukaemia, comprising 3 AGL (Figs. 1–4), 1 blastic crisis of CML, and 1 ALL (Figs. 6 and 7). In all 5 cases in which virus-like particles were seen, the leucocytes were examined before the commencement of treatment. In one of the cases of AGL, cells were also examined on two subsequent occasions, once at a time of partial remission, and again during the terminal relapse phase of the illness; similar virus-like particles were seen on all three occasions. In each of these 5 cases, the virus-like particles were morphologically very similar, and were common, being present in many cell profiles. No particles of this type were observed in the many samples of normal peripheral blood and bone marrow leucocytes examined.

In all 5 cases, the virus-like particles were usually associated in groups of 30–50, and were of relatively uniform spherical outline; although some variation in size was apparent (80–140 mμ), most measured approximately 100 mμ. At high magnification (Fig. 4), most of the virus-like particles were seen to be made up of an outer membrane and an inner central dense zone which itself sometimes appeared to be surrounded by an ill-defined membrane. Occasional particles, however, displayed a central electron-lucent zone rather than the more usual central dense area. Moreover, a few particles were of less regular outline and appeared to show tail-like structures (Fig. 3) resembling those illustrated by Dmochowski, *et al.* [13]. In addition, groups of somewhat similar, but smaller and more pleomorphic, structures (Fig. 2) were also seen, but in smaller numbers.

These virus-like particles did not resemble pinocytotic or Golgi-associated vesicles, and were frequently seen to be quite distinct, topographically, from the Golgi apparatus (Fig. 1). Furthermore, the virus-like particles were smaller than the cytoplasmic granules, and in the cases of myeloid leukaemia (AGL and blastic crisis of CML) were not stained by the Graham and Karnovsky peroxidase technique [17] or by the Novikoff and Goldfischer variant [18] of this technique, while the typical cytoplasmic granules of myeloid blast cells were strongly reactive (Fig. 5), [19].

It should be stressed that no virus-like particles were seen extracellularly, and no evidence of budding at the cell surface was ever observed.

DISCUSSION

Although the basic nature of the intracytoplasmic particles we describe here must remain uncertain, they bear a striking resemblance to virus particles [20], and differ from those non-viral cytoplasmic structures which have frequently been confused with virus particles [7]. Thus, they are clearly distinguishable from nuclear pores sectioned tangentially and bear no resemblance to the vesicles frequently associated with the multivesicular bodies of leucocytes [19]. The particles we describe do not resemble pinocytotic or Golgi-associated vesicles and were frequently seen to be topographically quite distinct from the Golgi apparatus. Furthermore, the particles do not resemble the cytoplasmic granules of leukaemic cells and contain no peroxidase activity—an enzyme which can be demonstrated [19], in the great majority of the granules of myeloblasts by the Novikoff and Goldfischer technique [18].

The virus-like particles we describe in this paper resemble in certain respects those described both by Seman and Seman [14] and Dmochowski *et al.* [12, 13], but nevertheless, a number of differences are also apparent. Thus, the particles reported by Seman and Seman resemble those described here in being of similar size (70–100 mμ or more) and internal structure, in being located in the cytoplasmic matrix, and in not being seen budding from the external cell membrane. However, the particles of Seman and Seman do not appear to be characteristically grouped together in clumps, do not display a tail-like

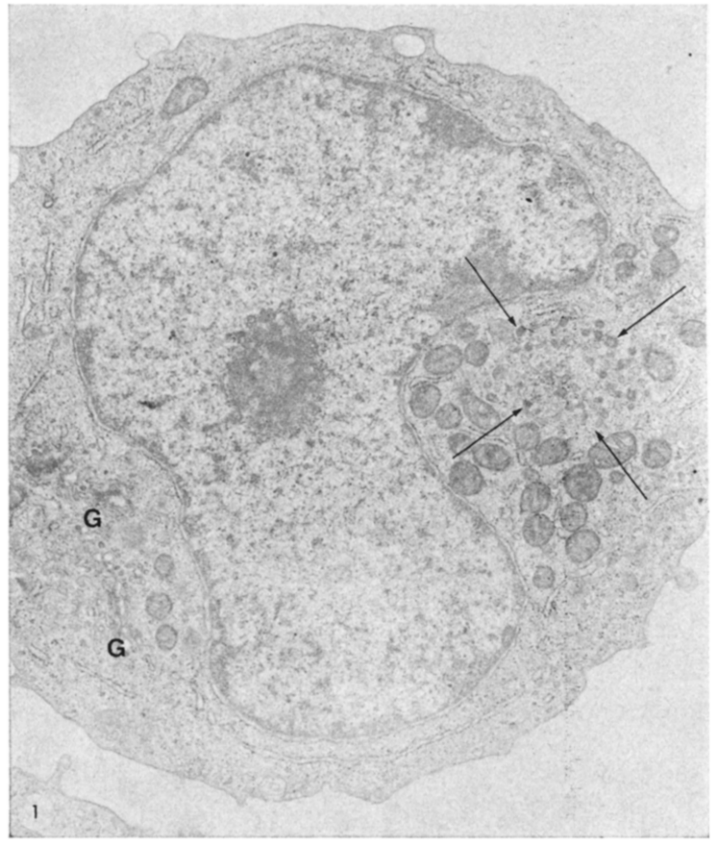


Fig. 1. *Untreated acute granulocytic leukaemia; peripheral blood* ($\times$ 21,000). *This blast cell contains a typical group of virus-like particles* ($\uparrow$). *In this case of AGL, similar virus-like particles were seen before treatment and twice subsequently, first when the patient was in partial remission, and later in complete relapse. These virus-like particles appear spatially well separated from the Golgi apparatus* (*G*), *and it seems unlikely, therefore, that they represent some form of Golgi-associated vesicle. This cell presents a completely undifferentiated appearance, and could not be assigned to any particular leucocyte series on the basis of its morphology alone; however, since many typical granulocytic cells were present in this patient, the cell is, presumably, a myeloblast.*

(to face p. 560)

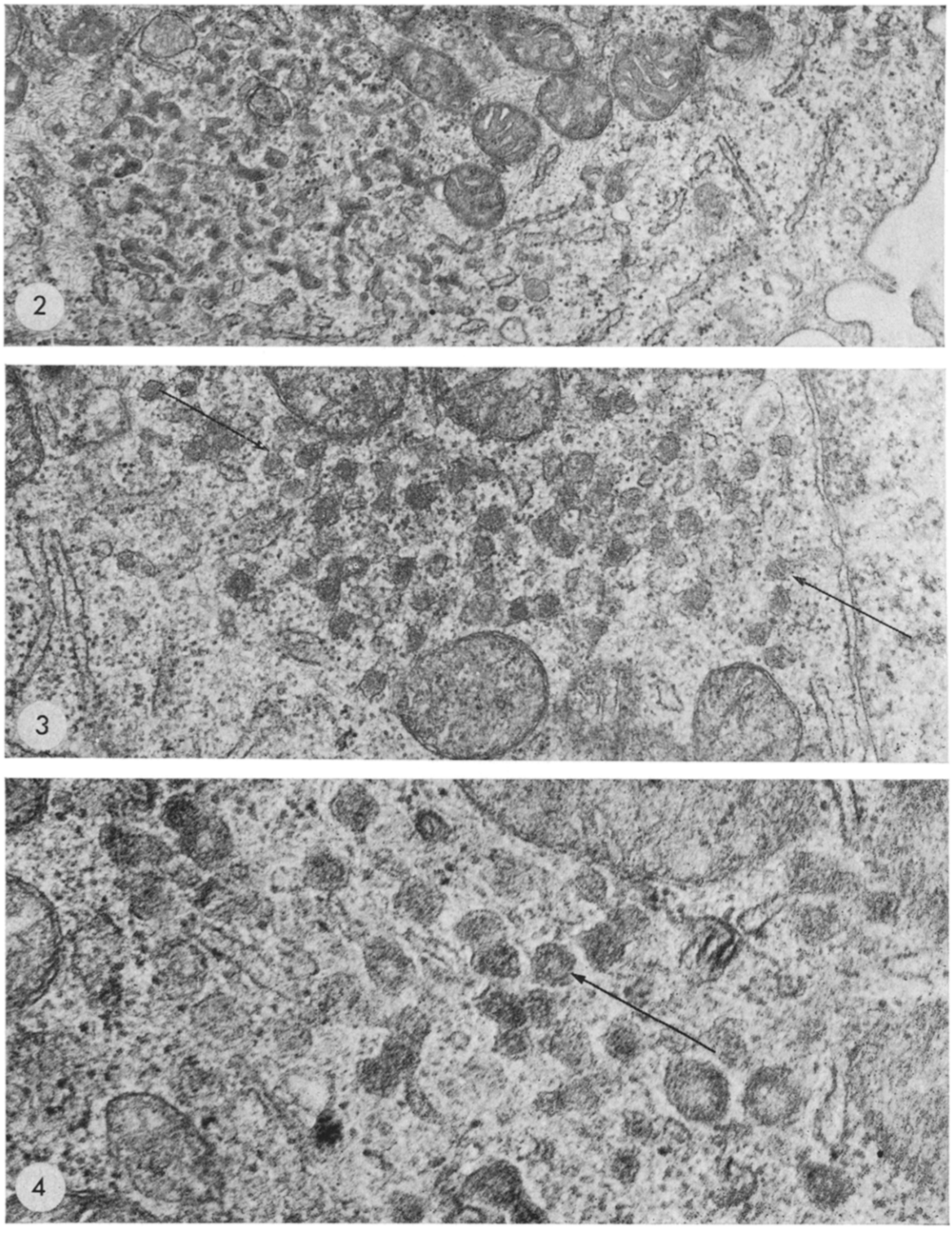


Fig. 2, 3 *and* 4 *are all portions of peripheral blood cells from the case of AGL illustrated in Fig.* 1. *The cells of Fig.* 2 *and* 3 *were obtained before treatment, while that of Fig.* 4 *was obtained during the terminal relapse phase of the illness.*

Fig. 2. (×25,000) *The particles illustrated here are appreciably smaller and more pleomorphic than the virus-like particles shown in Fig.* 3 *and* 4, *but are grouped together in a similar fashion. Such groups of particles were less commonly seen than the groups of virus-like particles, but were nevertheless not rare. The nature and significance of these particles is uncertain, but they may represent precursor forms of the virus-like particles illustrated in the other figures.*

Fig. 3. (×40,000) *A group of virus-like particles resembling those seen in Fig.* 1 *is illustrated at higher magnification. The particles are seen to be of relatively uniform size (approx.* 100 *mμ) and shape, and consist of an outer membrane and a central ill-defined denser area. In addition, a few of the particles show tail-like structures* (↑) *resembling those illustrated by Dmochowski* et al. [13].

Fig. 4. (×75,000) *Another group of virus-like particles is illustrated at high power. The individual particles show more variation in size and shape than is usually seen either before or after the commencement of therapy. The average-sized particles are again approx.* 100 *mμ in dia, but other particles range in size from* 80 *to* 140 *mμ. In some of the particles (e.g.* ↑), *the central dense area also appears to be surrounded by a membrane, but this is not clearly resolved. A few of the particles display a central lucent zone rather than the usual central dense area.*

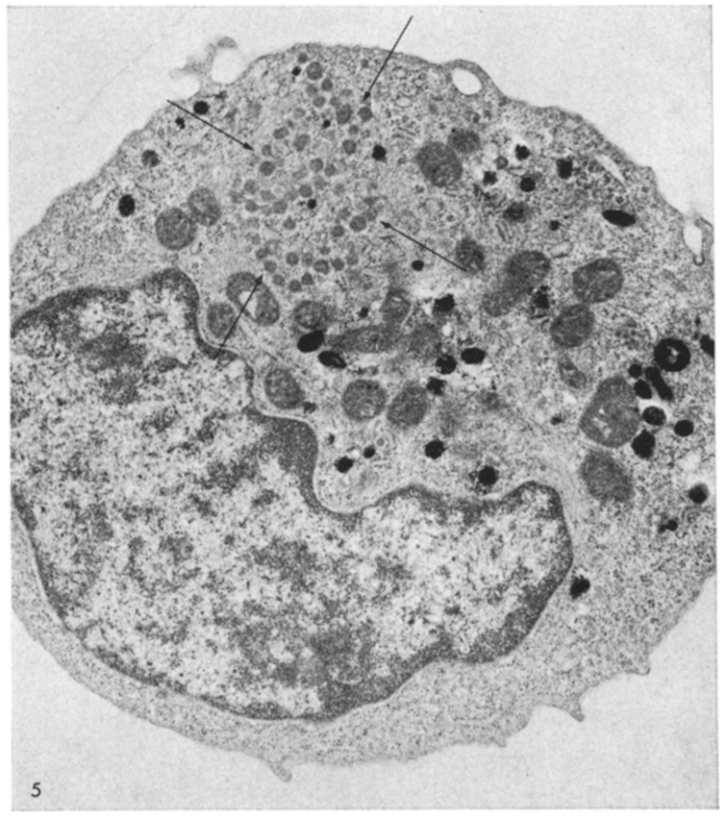


Fig. 5. *Acute granulocytic leukaemia; peripheral blood; Novikoff and Goldfischer technique* (× 21,000). *This primitive myeloid cell contains many cytoplasmic granules, all of which display strong cytochemical positivity. The typical group of virus-like particles* (↑) *is seen to be cytochemically unreactive; it is therefore unlikely that these particles represent any atypical granule form, since all the cytoplasmic granules of myeloblasts are stained by the Novikoff and Goldfischer technique* [19].

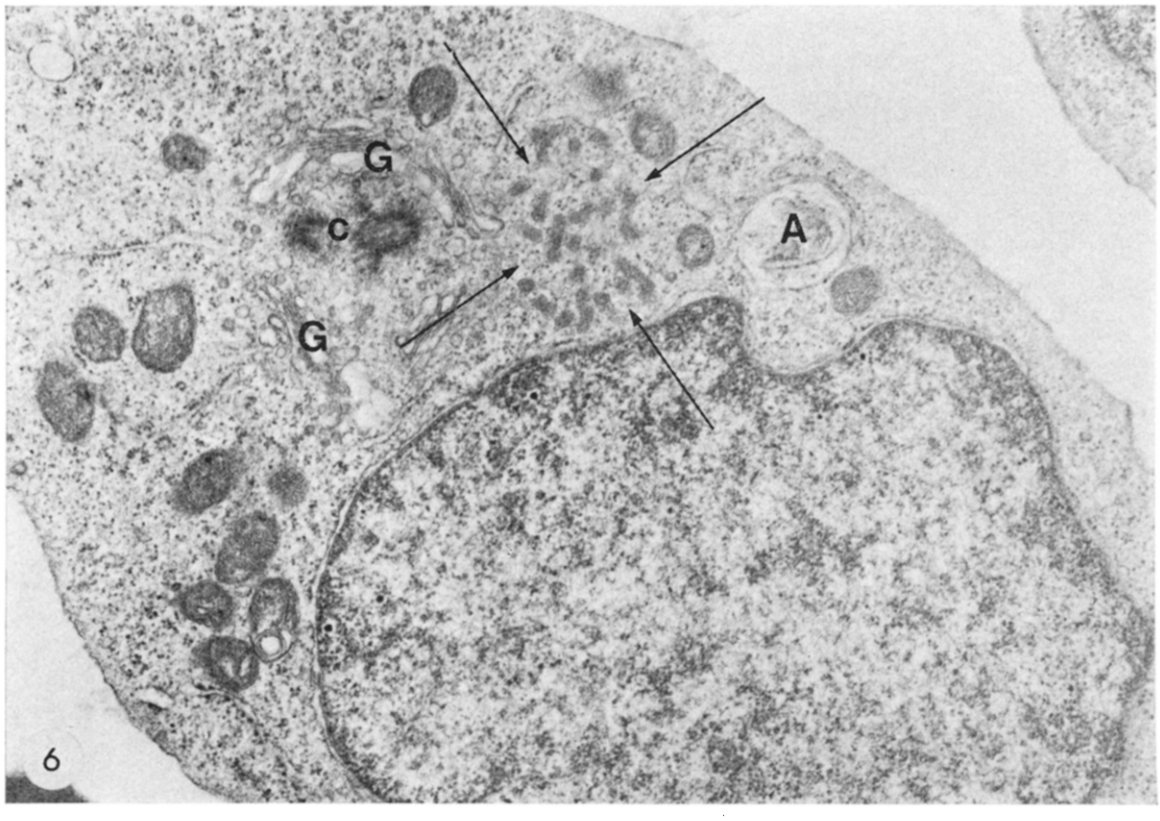


Fig. 6. *Untreated acute lymphoblastic leukaemia; peripheral blood* (× 22,000). *This blast cell contains a group of virus-like particles* (↑ *s*) *which closely resembles that in Fig.* 1. *Although situated close to the Golgi apparatus* (*G*) *and its two centrioles* (*c*), *the virus-like particles do not resemble Golgi-associated vesicles. This cell presents a very primitive appearance, but is presumed to be a lymphoblast since the patient had typical ALL. A probable autophagic vacuole* (*A*) *is present, but the morphology of the cell is otherwise unremarkable.*

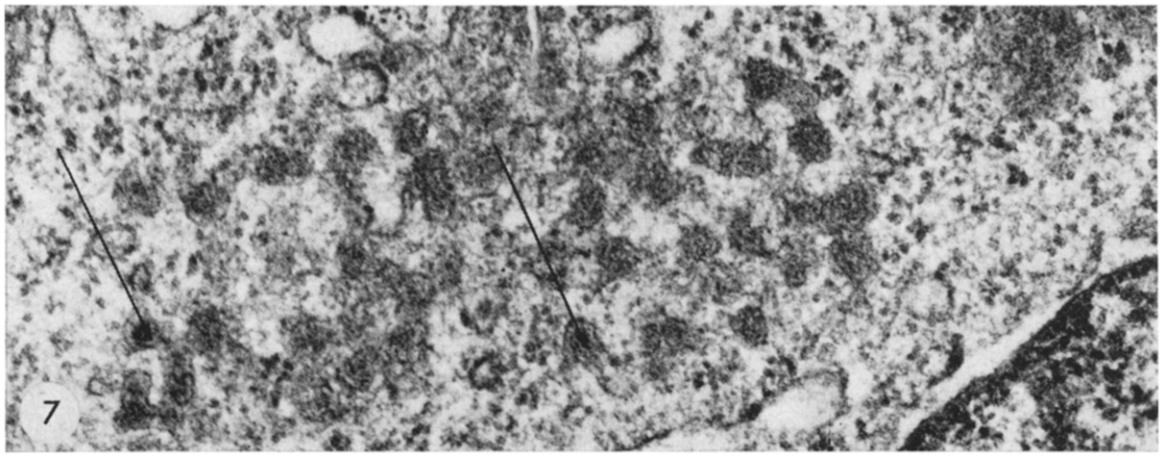


Fig. 7. *Untreated acute lymphoblastic leukaemia; peripheral blood* (× 60,000). *A further example of a group of particles from a case of acute lymphoblastic leukaemia is illustrated at higher magnification. The fine structure of the particles closely resembles that seen in the cases of myeloid leukaemia. The majority of particles again measure approx.* 100 *mμ in diameter, and possess an ill-defined central denser zone* (↑).

structure, and are seen budding from internal membranes.

Similarly, the virus-like particles described in the present study are of approximately the same size as those illustrated by Dmochowski *et al.* [12, 13] and contain a similar central dense zone while some also appear to possess similar tail-like structures. However, the particles described here are found free in the cytoplasm, while those of Dmochowski are located in intracytoplasmic vacuoles; moreover, no budding particles like those described by Dmochowski *et al.* were seen in the present study. The studies of Dmochowski *et al.* [12, 13] further differed from the present study in being largely confined to lymph node material from cases of ALL and CLL; these authors examined only seven bone marrow specimens (five of these were from cases of ALL), and do not appear to have examined peripheral blood leucocytes at all.

It has been established that C-type particles mature by budding from the cell surface. Therefore, if the intracytoplasmic structures we describe here in cells in which no budding particles could be seen are indeed an expression of viral information, they must represent an immature form of the virus. In their E.M. study of human cells infected with Rauscher leukaemia virus, Chandra *et al.* [21] demonstrated not only budding C-type particles, but also illustrated intracytoplasmic particles of somewhat variable morphology which they describe as virus forms of intermediate maturity. It is therefore conceivable that the particles we describe in this paper may also represent an intermediate form of the virus which in the human case cannot matuie to form typical complete C-type particles. The reason for this proposed failure of maturation is speculative, but it might result from the presence of incomplete viral genome in the host cells.

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REFERENCES

1. W. Bernhard and L. Gross, Presence de particules d'aspect virusal dans les tissus tumoraux de souris atteintes de leucémies induites. *C. R. Acad. Sci. (Paris)*, **248,** 160 (1959).
2. L. Dmochowski, C. E. Grey, J. A. Sykes, C. C. Schullenberger and C. D. Howe, Studies on human leukaemia. *Proc. Soc. exp. Biol. (N.Y.)* **101,** 686 (1959).
3. A. J. Dalton, J. B. Moloney, G. H. Porter, E. Frei and E. Mitchell, Studies on murine and human leukaemia. *Trans. Ass. Amer. Phycns* **77,** 52 (1964).
4. G. H. Porter, A. J. Dalton, J. B. Moloney and E. Z. Mitchell, Association of electron-dense particles with human acute leukaemia. *J. nat. Cancer Inst.* **33,** 547 (1964).
5. P. H. Levine, J. S. Horoszewicz, J. T. Grace, L. S. Chai, R. R. Ellison and J. F. Holland, The clinical status of leukaemia patients and virus-like particles in the plasma. *Ninth International Cancer Congress, Tokyo*, p. 270 (1966).
6. L. Gross, *Oncogenic Viruses.* Pergamon Press, Oxford (1970).
7. F. Haguenau, "Virus-like" particles as observed with the electron microscope. Chapt. 23—Ultrastructure of animal viruses and bacteriophages. In *Ultrastructure in Biological Systems* Vol. 5. Academic Press, London (1973).
8. H. Braunsteiner and F. Pakesch, Elektronenmikroskopische Untersuchungen der Granula menschlicher Leukozyten. *Acta haemat. (Basel)*, **17,** 136 (1957).
9. M. Bessis and J. P. Thiéry, Etudes au microscope electronique sur les leucémies humaines. I. Les leucémies granulocytaires. *Nouv. Rev. franc. Hémat.* **1,** 703 (1961).
10. M. Bessis and J. P. Thiéry, Etudes au microscope electronique sur les leucemies humaines. II. Les leucémies lymphocytaires. Comparison avec la leucémie de la souris de souche AK. *Nouv. Rev. franc. Hémat.* **2,** 387 (1962a).
11. M. Bessis and J. P. Thiéry, Etudes au microscope electronique des hemosarchomes humains. III. Leucémies à cellules-souches, erythrémies reticulolymphosarcomes maladie de Hodgkin, plasmocytomes. *Nouv. Rev. franc. Hémat.* **2,** 577 (1962b).

12. L. Dmochowski, Y. G. Taylor, G. E. Grey, E. Designer, D. A. Dreyer, T. A. Sykes, P. L. Langford, T. Roges, C. C. Schullenberger and C. D. Howe, Viruses and mycoplasma (PPLO) in human leukaemia. *Cancer (Philad.)* **18,** 1345 (1965).
13. L. Dmochowski, T. Yumoto, C. E. Grey, R. L. Hales, P. L. Langford, H. G. Taylor, E. J. Freireich, C. C. Schullenberger, J. A. Shively and D. D. Howe, Electron microscopic studies of human leukaemia and lymphoma. *Cancer (Philad.)* **20,** 760 (1967).
14. G. Seman and C. Seman, EM search for virus particles in patients with leukaemia and lymphoma. *Cancer (Philad.)* **22,** 1033 (1968).
15. F. G. J. Hayhoe, D. Quaglino and R. Doll, The cytology and cytochemistry of acute leukaemias. A study of 140 cases. *M.R.C. Special Report Series*, No. 304. Her Majesty's Stationery Office, London (1964).
16. J. C. Cawley and F. G. J. Hayhoe, The inclusions of The May–Hegglin anomaly and Döhle bodies of infection: an ultrastructural comparison. *Brit. J. Haemat.* **22,** 491 (1972).
17. R. C. Graham and M. J. Karnovsky, The early stages of absorption of horseradish peroxidase in the proximal tubules of mouse kidney; ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14,** 291 (1966).
18. A. B. Novikoff and S. Goldfischer, Visualization of microbodies for light and electron microscopy. *J. Histochem. Cytochem.* **16,** 507 (1968).
19. J. C. Cawley and F. G. J. Hayhoe, *Ultrastructure of Haemic Cells*. Saunders, London (1973).
20. A. J. Dalton and F. Haguenau, Ultrastructure of animal viruses and bacteriophages. *Ultrastructure in Biological Systems*. Vol. 5. Academic Press, London (1973).
21. S. Chandra, R. Stephans, B. S. Wright, W. Karol, I. Zelljadt, and E. M. Jenson, Type-C particles in human tissues. II. Electron microscopic study of embryonic cultures infected with a murine leukaemia virus. *Int. J. Cancer*, **6,** 46 (1970).

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Feulgen Hydrolysis of Normal and Leukemic Blood Cells

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Abstract—*The hydrolysis kinetics of the Feulgen reaction has been followed on different stages of maturation of leucocytes from normal and pathological origin. The data suggest that either the physico-chemical modifications of the nuclear DNA–protein complex of these elements are negligible or that the analysis of Feulgen hydrolysis kinetics is insufficiently sensitive to bring them to light.*

INTRODUCTION

THE KINETICS of chromatin DNA acid hydrolysis at room temperature can be followed by Feulgen staining, as has been shown by Jordanov [1]. The kinetics of hydrolysis are probably dependent upon the physico-chemical characteristics of the DNA–protein complex. Differences between the hydrolysis curves of nuclear DNA in young embryos and in well differentiated cells have been reported (Agrell and Berqvist [2]; Brachet and Preumont [3]. The kinetics of the Feulgen reaction in normal and cancer cells are different reflecting, according to Sandritter [4] and Sandritter *et al.* [5], different proportions of eu- and heterochromatin in the nuclei of normal and malignant cells. Eu- and heterochromatin isolated from calf thymus behave differently when subjected to acid hydrolysis. It has also been suggested that the nuclei might contain two kinds of DNA which have different stability and unequal resistance to acid hydrolysis (Sampson *et al.* [6]; Böhm and Sandritter [7]; Sampson and Davies [8].

On the other hand, Eberhard and Mazia [9] have shown that the differences in resistance to acid hydrolysis in early and late sea urchin embryos disappear if the hydrolysis is carried out on purified DNA: this demonstrates the importance of the proteins associated with the DNA in influencing the hydrolysis.

We hoped that a study of the kinetics of Feulgen reaction might give us direct information about the activity of the genome. This technique has been applied to the study of leucocytes and those in various haematological diseases; different stages of maturation in hemopoietic tissue have been examined. Our purpose was to test the sensitivity and specificity of the Feulgen hydrolysis curves.

MATERIAL AND METHODS

Bone marrow and peripheral blood smears from 2 normal subjects, from 2 cases of acute myeloblastic leukemia (AML), from 4 cases of acute lymphoblastic leukemia (ALL) and from a patient with infectious mononucleosis from which we have examined peripheral lymphomonocytes at different stages of the disease, were prepared. Blood smears have also been prepared at 1, 4, 8, 12, 24, 36, 48, 60, 72 hr after incubation with phytohaemagglutinin (PHA) treatment (2 normal subjects were examined). In one AML patient we studied the effects of two doses of methotrexate (MTX) (totalling 0·5 mg/kg) given 62–72 hr before taking the cell samples. Three L-asparaginase sensitive ALL cases were studied 18 hr, 6 and 15 days after administration of the enzyme (6·000 UI/m^2 daily).

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The smears were fixed with acetic Zenker; they were then hydrolysed with HCl 1N at 22°C for various times and stained with the Schiff reagent. Nuclear staining intensity was measured with a Barr and Stroud microdensitometer. The instrument error was about 3% and the spread about the mode for a normal leucocyte population at different times of hydrolysis is 7%.

RESULTS

Figures 1 and 2 show the hydrolysis kinetics of different blood elements in the same normal case: marrow myeloblasts, myelocytes, granulocytes, orthochromatic erythroblasts and lymphocytes (Fig. 1) peripheral granulocytes and lymphocytes (Fig. 2). It is clear that no significant differences exist between cells which differ both cytologically and functionally. During PHA lymphocyte activation (Fig. 3), the kinetics of Feulgen hydrolysis did not vary significantly. The same kinetics were found for pheripheral blood lymphomonocytes in infectious mononucleosis, at different clinical stages.

Among the acute leukemias studied, no significant differences in the kinetics of Feulgen hydrolysis were observed when comparing marrow and pheripheral leucocytes from the same patient. After L-asparaginase chemotherapy, no differences were observed in three cases of ALL. In one AML case treated by MTX (Fig. 4), one important shift to the right of the Feulgen hydrolysis biphasic curve has been observed: it implies a higher resistance of DNA to acid hydrolysis.

The marrow proliferative and non-proliferative blastic elements present the same modifications (Fig. 4). The same case showed a rise in both the thymidine labelling index and in ^3H actinomycin binding (Pileri *et al.* [12], Brachet *et al.* [10].).

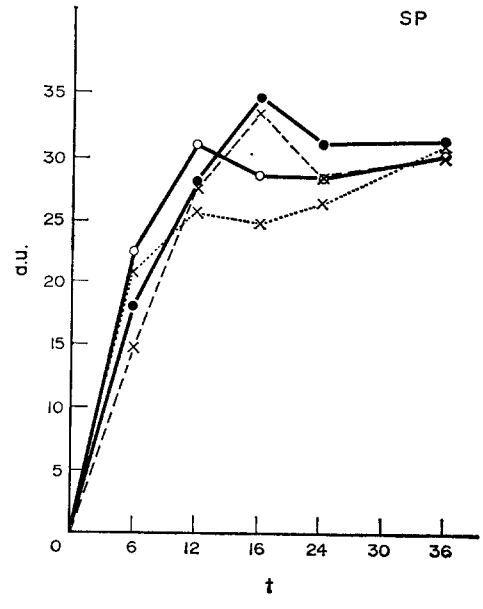


Fig. 2. Two normal subjects
●—● *Case 1* ○—○ *Case 2* *Lymphocytes*
× --- × *Case 1* × . . . × *Case 2* *Granulocytes*
SP: peripheral blood, AU: Feulgen arbitrary units, t: time of hydrolysis (hr).

DISCUSSION

It was hoped that the study of the Feulgen hydrolysis kinetics would provide specific information about the association between DNA and proteins, and possible changes at

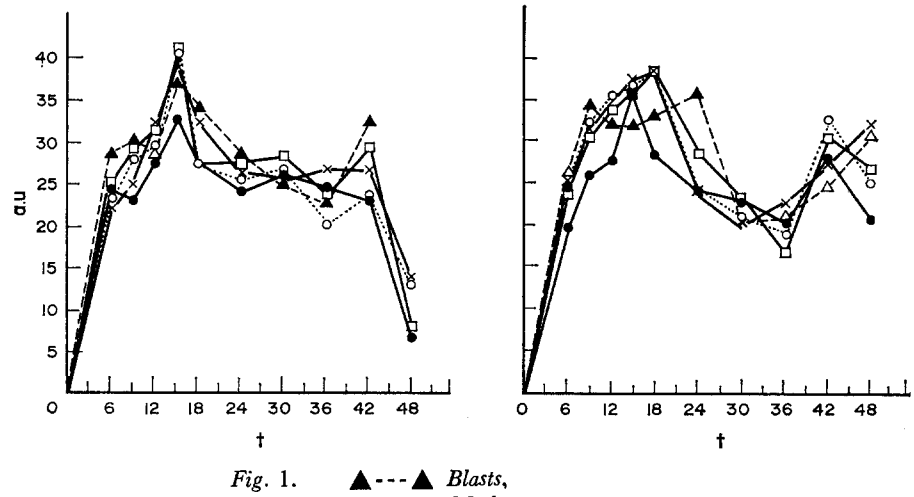


Fig. 1. ▲---▲ *Blasts,*
× —— × *Myelocytes.*
○ . . . ○ *Lymphocytes.*
●—● *Erythroblasts.*
□—□ *Granulocytes.*
AU: Feulgen arbitrary units, t: time of hydrolysis (hr).

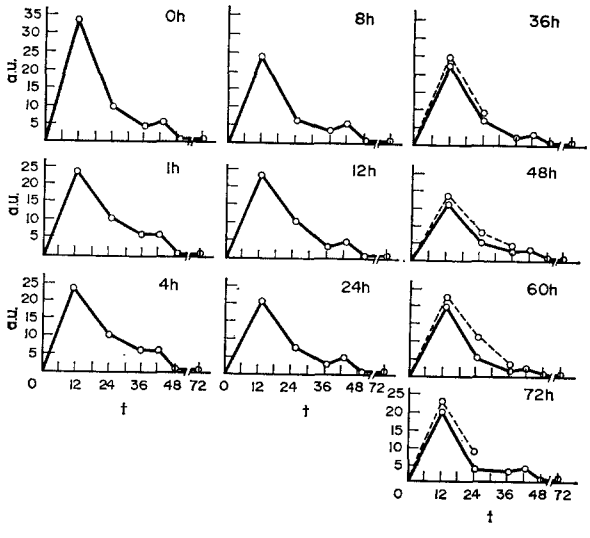


Fig. 3. Feulgen kinetic of normal lymphocytes cultured in presence of PHA.
●——● *Lymphocytes.*
○---○ *PHA activated lymphocytes.*
AU: Feulgen arbitrary units, t: time of hydrolysis (hr).

that level, possibly related to proliferation and differentiation in normal cells as well as functional abnormalities in malignant cases. This study, which was carried out on very heterogeneous populations of the normal and the leukemic hemopoietic system, failed to show any significant differences between the different populations. This is true for both the lymphocytic and granulocytic peripheral elements and for the cultured lymphocytes before and after PHA treatment, that is to say, for cells known to display different levels of gene activity (Cooper [11]). The data suggest that either the physico-chemical modifications of the DNA–protein complex under such conditions of proliferation and differentiation are of negligible importance, or that the analysis of Feulgen hydrolysis kinetics is not sensitive enough to bring them to light. The only significant variation found was in a single AML case (treated with two high doses of MTX which removed 99% of the blast population): in that patient, the surviving population showed a real gene activation, as was seen in our previous studies on their proliferative capacity and actinomycin binding (Pileri *et al.* [12]), which suggest a re-entry in the cycle of the blast elements which have survived the cytostatic treatment (Gabutti *et al.* [13]). Actinomycin binding seems to be a more accurate technique for the study of functional nuclear modifications in intact cells than the analysis of Feulgen hydrolysis kinetics. The results obtained by the former method can be compared with those obtained by studying the transcription of chromatin templates in cell free systems.

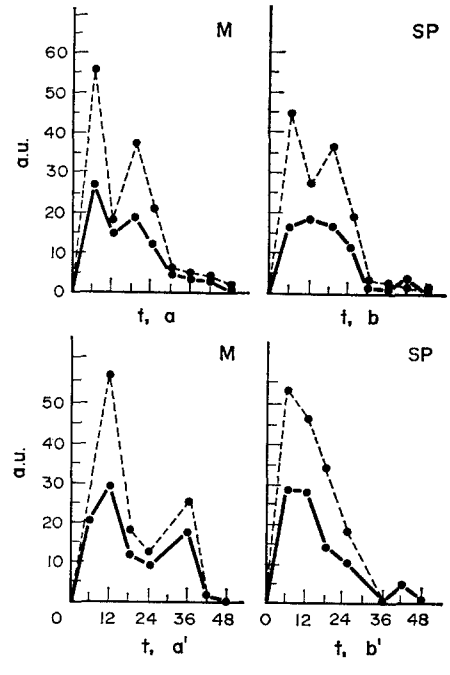


Fig. 4. Feulgen kinetics of acute myeloblastic leukemia
a, b: untreated.
a', b': MTX treated.
M: marrow, SP: peripheral blood, AU: Feulgen arbitrary units, t: time of hydrolysis (hr).

REFERENCES

1. I. J. Jordanov, On the transition of desoxyribonucleic acid to apurinic acid and the loss of the latter from tissues during Feulgen reaction hydrolysis. *Acta histochem.* **15,** 135 (1963).
2. I. Agrell and H. A. Berqvist, Cytochemical evidence for varied DNA complexes in the nuclei of undifferentiated cells. *J. Cell. Biol.* **15,** 604 (1962).
3. J. Brachet and A-M. Preumont, Nouvelles techniques de détection des acides désoxyribonucléiques: quelques résultats préliminaires. *Ann. Histochem.* **11,** 157 (1966).
4. W. Sandritter, DNA content of tumors. Cytophotometric measurements. *Europ. J. Cancer* **1,** 303 (1965).
5. W. Sandritter, K. Jobst, L. Rakow and K. Bosselman, Zur kinetic der Feulgen reaction bei verlängerter Hydrolysezeit. Cytophotometrische Messungen im sichtbaren und ultravioletten Licht. *Histochemie* **4,** 420 (1965).

6. M. Sampson, A. Katoh, Y. Hotta and H. Stern, Metabolically labile deoxyribonucleic acid. *Proc. nat. Acad. Sci.* (*USA*) **50,** 459 (1963).
7. N. Böhm and N. Sandritter, Feulgen hydrolysis of normal cells and mouse ascites tumor cells. *J. Cell. Biol.* **28,** 1 (1966).
8. M. Sampson and D. D. Davies, Synthesis of a metabolically labile DNA in the maturing root cells of *Vicia fabia*. *Exp. Cell. Res.* **43,** 669 (1966).
9. A. Eberhard and D. Mazia, Conditions for extractions of cytoplasmic DNA from embryos of a Sea Urchin. *Biochim. Biophys. Comm. Res.* **21,** 460 (1965).
10. J. Brachet, N. Hulin, P. Masera and A. Pileri, L'activité de la chromatine des leucocytes normaux et leucémiques. *C.R. Acad. Sci.* (*Paris*), **271,** 2070 (1970).
11. H. L. Cooper, Ribonucleic acid metabolism in lymphocytes stimulated with phytohaemagglutinin. II. Rapidly synthesized ribonucleic acid and the production of ribosomal ribonucleic acid. *J. biol. Chem.* **243,** 34 (1968).
12. A. Pileri, P. Masera, G. Garbarino and N. Hulin, Actinomycin binding in human myeloid leukaemia. *Acta haemat.* (In press).
13. V. Gabutti, A. Pileri, R. P. Tarocco, F. Gavosto and E. H. Cooper, Proliferative potential of out-of-cycle leukaemic cells. *Nature* (*Land*). **224,** 375 (1969).

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Effects of Anticancer Agents on the Respiration of Isolated Mitochondria and Tumor cells*

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Abstract—*A number of anticancer agents of high efficiency and yet considerable toxicity, interact with the bioenergetics and ion transport of not only the target tumor cells, but those of the host organs as well. The possibility that there is a generalized site of toxicity of such agents with the host organ, particularly the heart and liver, has encouraged us to make a more detailed study of the site of action of respiratory inhibition by a variety of anticancer drugs selected for this purpose.*

INTRODUCTION

THE KNOWLEDGE of the effects on respiration of new anticancer drugs is desirable on several grounds. Compounds which, due to their efficiency and tolerable toxicity, reach the clinical trial phase of anticancer chemotherapy screening programs, may exhibit toxicity or biochemical side effects due to the action of the drug on bioenergetics and ion transport. Additionally, many drugs with strong effects on respiration and ion transport will appear as effective anticancer agents in initial screening involving intraperitoneal injection of the drug in mouse ascites tumors and subsequent observations of the effects of the drug on tumor growth, but will fail subsequent toxicity tests due to lack of specificity.

The hypothesis that a particular site in the respiratory chain, namely the rotenone-sensitive site, is a characteristic site for anticancer drugs which may act on the respiratory chain, has been put forward by one of us, [1] and further studies have shown that the agent, VM 26, is an inhibitor of NADH-linked respiration [2]. This contribution demonstrates the effects of a number of cancerostatic agents, obtained from the National Cancer Institute, at NIH, Bethesda, upon the respiration of avian cardiac mitochondria, rat liver mitochondria, and of Ehrlich ascites tumor cells. The redox states of cytochrome *b* and pyridine nucleotide were also measured.

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MATERIAL AND METHODS

Rat liver, rat heart and pigeon heart mitochondria were isolated by the method of Schneider [3] and Chance and Hagihara [4], respectively. Ehrlich ascites cells were collected from six-day Ehrlich ascites tumors, (Lettré hyperdiploid strain or a hypertetraploid strain) and hemoglobin-free suspensions were prepared as described by Chance and Hess [5]. The mixed population of leucocytes and lymphocytes was isolated by differential centrifugation

from the buffy coat of 2 l of citrated pig blood. Oxygen uptake was measured with a Clark type oxygen electrode at 22°C in a 1 ml chamber. For each drug concentration, the rate of O_2 uptake was calculated in State III (+ATP) and on subsequent depletion of ADP (State IV). The respiratory control ratio was calculated as the ratio of oxygen uptake in State III to that in State IV. The ADP : O ratio was calculated by determination of the absolute number of ADP molecules added to the mitochondrial suspension, and subsequent division by the number of oxygen atoms taken up by the mitochondria in State III respiration. An assay medium composed of 225 mM sucrose, 20 mM KCl, 7 mM $MgCl_2$, 10 mM Tris–HCl, and 5 mM Tris–Pi at pH 7·2 was used for isolated mitochondria, and a phosphate saline medium [5] was used for the assay of respiration of the Ehrlich ascites tumor cells and the leucocytes. The redox states of mitochondrial pyridine nucleoetides and cytochrome *b* were measured with a dual wavelength spectrophotometer, as described by Chance and Williams [6]. The anticancer drugs, which were supplied by the Drug Evaluation Branch of the National Institute of Cancer, (Bethesda), were used in concentrated stock solutions in water, ethanol, or N,N,dimethylformamide and added in microliter quantities. Solvents were used as controls. Substrates, salts and cofactors were purchased from Sigma Company. Mitochondrial protein was determined by the biuret reaction [7]. In all experiments measuring oxygen uptake in mitochondria and Ehrlich Ascites cells, an 8 min preincubation period with the drug was employed, since the activities of the 4 agents are time-dependent. The capacity of the drugs to reverse oligomycin inhibition was measured by first inhibiting mitochondrial respiration with a concentration of 1 μg oligomycin per mg of mitochondrial protein. Addition of the relevant drug may then lead to release of the inhibition, which is readily observed as an increase of O_2 uptake as measured by the O_2 electrode.

RESULTS

A. *Comparison of the effects of a number of drugs on isolated mitochondria and Ehrlich Ascites cells*

Table 1 shows the relative inhibitory activities of a variety of anticancer agents. The first 8 agents are active, inhibiting mitochondrial respiration in the presence of ADP (State 3 respiration [8]), but the extents of inhibition and the concentrations required for 50% inhibition vary widely over the series. VM-26 and ellipticene are the most active inhibitors, while the last 4 compounds of the series do not show activity on respiration. Between these two extremes, there is a group represented by adriamycin, Me-CCNU, CCNU, daunomycin, and ethidium bromide, which are able to inhibit nearly 100% of mitochondrial respiration at 1 mM concentration, and a group

Table 1. *Effects of anticancer agents on the respiration of isolated mitochondria and Ehrlich ascites cells*

Anticancer agent	Inhibition of mitochondrial State 3 respiration* Concentration	inhibition	Reversal of Oligomycin Inhibition	Inhibition of endogenous respiration of Ehrlich ascites cells† Concentration	Inhibition
	μM	%		nmole/10^6 cells	%
VM-26	12·5	50	no	1	50
Ellipticene	50	50	no	2·5	50
Adriamycin	200	50	yes	12·5	50
Me-CCNU	300	50	no	5	50
CCNU	400	50	no	20	50
Daunomycin	400	50	yes	40	50
Ethidium bromide	400	50	yes	40	30‡
Diaminechloroplatinum	800	40‡	yes	40	0
Picolinaldehydethiosemicarbazone	800	25‡	—	40	0
IRCF-159	800	0	—	40	10‡
Emetine	800	0	—	40	0
Hydroxyurea	800	0	—	40	0
Cordycepin	800	0	—	40	0

*0·4 mg/ml of mitochondrial protein. Pigeon heart mitochondria. Glutamate plus malate, 6 mM; ADP, 0·3 mM.
†30×10^6 cells/ml Lettre hyperdiploid strain.
‡Greatest degree of inhibition obtainable.

comprising diaminochloroplatinum and picolinaldehyde thiosemicarbazone, with which it is impossible to achieve complete inhibition, even at very high concentrations. Some of the compounds are able to release the respiratory inhibition caused by oligomycin, which defines them as acting on the mitochondrial energy transfer pathway closer to the respiratory chain than the site of oligomycin inhibition (thought to be at the ATP synthetase enzyme). Table 1 also shows the effects of these anticancer agents on the respiration of isolated Ehrlich ascites cells, which can be compared with the action on mitochondria. It appears that there is a good correlation between the relative potencies of the anticancer agents with respect to their respiratory inhibitory properties in mitochondria and in ascites cells. There are only two exceptions; Me–CCNU appears to be more active than adriamycin in tumor cells, but less active in mitochondria, and ICRF 159, which is inactive in isolated mitochondria, has a small degree of activity in isolated tumor cells. It is not possible to correlate the reversal of the oligomycin inhibition with the ability of the drugs to inhibit mitochondrial (and cellular) respiration, except in the cases of ethidium bromide and diaminochloroplatinum, which show this activity and also show poor inhibitor properties. The reversal of oligomycin inhibition is an activity which is characteristic of mitochondrial uncouplers

From the group of compounds described in Table 1, the 2 most active, VM-26 and ellipticene, and 2 of intermediate potency, daunomycin and adriamycin, were selected for further study.

B. *The effects of daunomycin and adriamycin on isolated mitochondria*

Figures 1(a, b, and c) show the effects of these 4 agents on mitochondrial oxygen uptake in State 3 (Fig. 1a), State 4 (oxygen uptake on depletion of ADP [8]) (Fig. 1b) and the ADP:O ratio, (which acts as a measure of oxidative phosphorylation). In Fig. 1 (d e and f), respectively, are shown the effects of the agents on the same parameters with succinate as

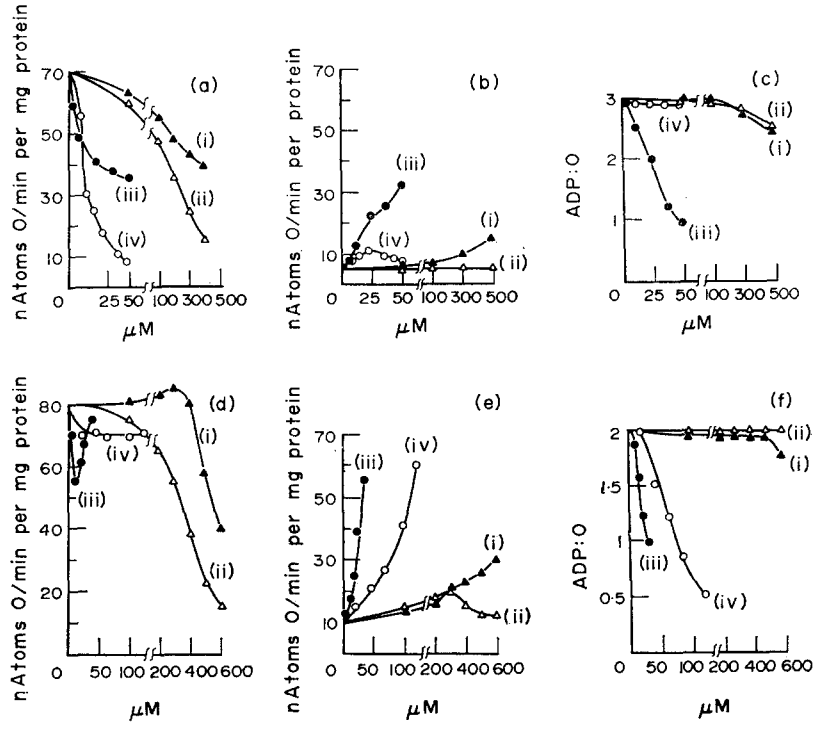


Fig. 1. *(a b and c) Effect of increasing concentrations of daunomycin (i), adriamycin (ii), ellipticene (iii) and VM* 26 *(iv) on mitochondrial respiration in State* 3 *(a), State* 4 *(b), and the ADP* :0 *ratio (c), employing glutamate and malate as substrates. (d, e and f). Effect on increasing concentrations of daunomycin (i), adriamycin (ii), ellipticene (iii), and VM* 26 *(iv) on mitochondrial respiration in State* 3 *(d) and State* 4 *(e) and the ADP : O ratio (f), employing succinate as substrate.*

In all these experiments, 0·8 *mg/ml of mitochondrial protein (rat liver) was used,* 22°*C, glutamate and malate* (5 *mM*), 0·3 *mM ADP addition. Preincubation time* 8 *min prior to addition of ADP. From Fig. (a) and (b) and Fig. (c) and (d), the effect of the drugs on the respiratory control, i.e. the ratio of respiration in State* 3 *to that in State* 4, *can be deduced.*

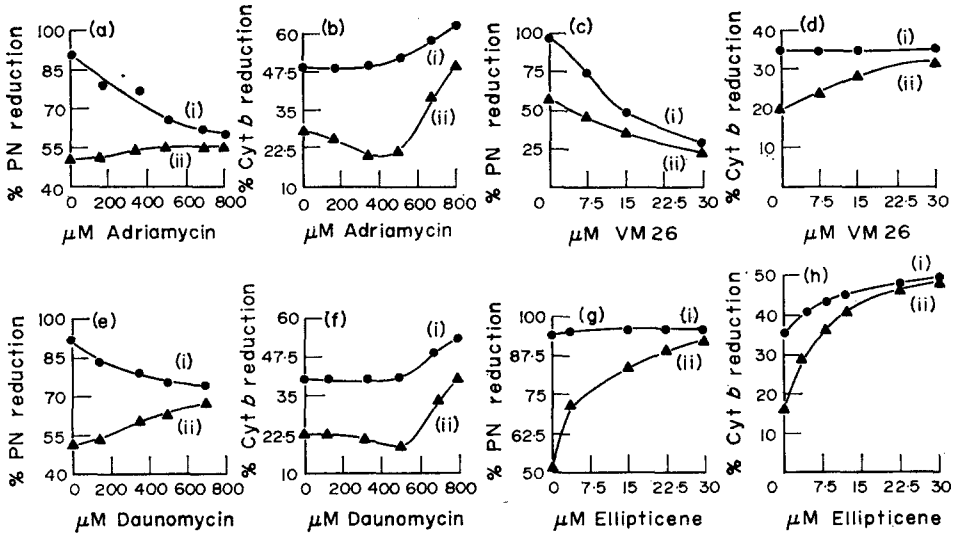


Fig. 2. Effect of increasing concentrations of adriamycin, VM 26, daunomycin and ellipticene on the redox state of the pyridine nucleotides and cytochrome b *in State 4 (i) and in State 3 (ii). Rat liver mitochondria were used at 0·8 mg/ml. 5 mM β-hydroxybutyrate as substrate. 0·3 mM ADP addition. One hundred per cent reduction measured by anaerobiosis and 0% reduction was obtained by ADP addition to mitochondria respiring with endogenous substrate [7]. The drug was added after the first ADP cycle, the resultant artifact attenuated, and a preincubation time of 3 min allowed prior to the second ADP addition.*

substrate. In order to pinpoint the site of action of these agents in the respiratory chain, their effects on the redox changes of mitochondrial pyridine nucleotides and cytochrome *b* were measured. The results of these experiments are shown in Fig. 2.

Daunomycin and adriamycin were able to inhibit State 3 respiration to different, but considerable, extents, at concentrations between 300 and 500 μM with both substrates. They had little effect on State 4 respiration with either substrate. They also had little or no effect on oxidative phosphorylation, as demonstrated by the ADP : O ratios described in Fig. 1(c) and 1(f). In the redox experiments, adriamycin was found to cause the oxidation of pyridine nucleotides in State 4 with a small reduction in State 3, These changes were accompanied at high concentrations by a reduction of cytochrome *b* in States 3 and 4. Similar results were obtained with daunomycin. These results are illustrated in Fig. 2(a, b) and Fig. 2(e) F for adriamycin and daunomycin, respectively. It is also interesting to note that, at intermediate concentrations of daunomycin and adriamycin, the magnitude of the redox change associated with the State 4 to 3 transition increases for cytochrome *b*, but decreases for the pyridine nucleotides.

C. *Cardiac toxicity of adriamycin*

Adriamycin has been reported in clinical studies to show occasional cardiac toxicity

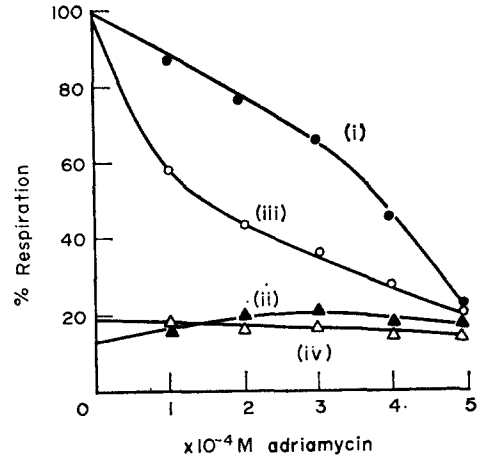


Fig. 3. Effect of increasing concentrations of adriamycin in the respiration of isolated rat liver mitochondria in State 3 (i) and State 4 (ii) and rat heart mitochondria in State 3 (iii) and State 4 (iv). Glutamate and malate as substrate 5 mM, 0·3 mM ADP addition, 8 min preincubation prior to ADP addition. Six-tenths mg mitochondrial protein per ml was employed for rat liver mitochondria and 0·2 mg mitochondrial protein for the rat heart mitochondria. One hundred per cent respiration was 42 n atoms oxygen/min.

[9–11]. It was thought interesting to determine whether the cardiac toxicity of adriamycin could be related to its effects on respiration. Figure 3 compares the effects of increasing concentrations of adriamycin on the respiration of isolated rat heart and rat liver mitochondria. It was concluded that no significant differences in sensitivity could be observed between the two types of mitochondria,

which were studied at equal cytochrome *a* concentrations in order that the results be comparable. The assumption of this approach is that the effective concentration of a respiratory inhibitor is related to the amounts of the respiratory carriers present. It should be noticed that cardiac toxicity (in the case of adriamycin, for example) should be subjected to further tests in the perfused organ which would give, as well as the necessary information on the toxicity, needed data on the tissue binding of adriamycin [12].

D. *Effects of VM-26 on isolated mitochondria*

VM-26 was found to inhibit mitochondrial respiration in State 3 with glutamate plus malate as substrate, but not with succinate (Fig. 1a, d), this inhibition being almost 100% at 50 μM. Stimulation of State 4 respiration by VM-26 occurred with succinate as substrate, but not with glutamate plus malate. As might have been expected from this data, the ADP : O ratio with glutamate and malate as substrate was unaffected by VM-26, but at 50–100 μM, succinate respiration was uncoupled (Fig. 1c, f). Increasing concentrations of VM-26 were found to produce an oxidation of the redox state of the pyridine nucleotides in States 3 and 4. The redox state of cytochrome *b* in State 3 was shifted to reduction, but no effect was observed on the redox state of cytochrome *b* in State 4 (Fig. 2c, d). These effects are difficult to interpret.

E. *Effects of ellipticene on isolated mitochondria*

Ellipticene was also found to inhibit State 3 respiration with glutamate and malate, but not with succinate, yet only partial inhibition was found with 50 μM ellipticene as compared with the total inhibition observed with VM-26 at this concentration. At concentrations greater than 12 μM, it was even found to stimulate State 3 respiration with succinate as substrate. Ellipticene was found to stimulate State 4 respiration and to uncouple oxidative phosphorylation with both substrates. In the redox experiments, it was found to cause a reduction of the pyridine nucleotides in State 3 with no effect on the State 4 redox state, but it caused a reduction of cytochrome *b* in both states (Fig. 2g, h). The reduction of both these carriers by ellipticene correlates with its uncoupling action mentioned earlier.

Therefore, from Fig. 1, we can conclude that daunomycin and adriamycin behave at high concentration as inhibitors of NADH-linked and succinate respiration, with very little uncoupling effect. Ellipticene appears to be an uncoupling agent with a concomitant inhibitory activity on succinate-linked respiration [1] and, at doses greater than 50 μM, is able to specifically uncouple succinate respiration.

One common feature of the data described in Fig. 2 is that all 4 compounds, at the concentrations required for maximum inhibition of respiration, decrease the magnitude of the redox change associated with the State 4–3 transition.

F. *Specificity of the four drugs for tumor cells*

The specificity of the respiratory inhibition of adriamycin, daunomycin and ellipticene in tumor cells was compared with that of VM-26 which has been shown to have a probably mechanism of action based on respiratory inhibition [2]. In order to accomplish this 2 methods were employed. First, the 4 drugs were injected intraperitoneally into mice bearing ascites tumor 6 days after transplantation of the tumor. The doses used ranged from one to several times the chemotherapeutic dose.* Samples of tumor cells were extracted 4 hr after the treatment and their respiration was measured in order to obtain the relationship between the respiratory inhibition and the chemotherapeutic dose. Secondly, the effects of the 4 drugs on the respiration of Ehrlich ascites tumor cell suspensions and of a mixed population of pig blood leucocytes and lymphocytes were compared. Cell concentrations were again adjusted in each case to yield equivalent respiration. Figure 4(a) shows the effect of increasing concentrations of the 4 drugs on the respiration of a suspension of Ehrlich ascites cells and it can be observed that both VM-26 and ellipticene produce a 90% inhibition of the respiration at 400 and 600 μM, respectively. Six mM concentrations of daunomycin and adriamycin are required to produce around 60% inhibition of respiration. Higher doses of these last 2 compounds did not show further inhibition. In comparison, when the effects of the drugs were tested in a mixed leucocyte/lymphocyte suspension, the results of Fig. 4(b) were obtained. Daunomycin had a greater effect at a lower concentration on leucocytes than on tumor cell respiration, while the

*The chemotherapeutic doses of adriamycin, daunomycin, ellipticene, and VM-26 are 5, 37, 64, and 90 mg/kg, respectively, optimum single intraperitoneal dose, when injected 1 day after transplanting the tumor (Personal communication, Dr. F. White, Head, Biochemistry Section, Drug Evaluation Branch, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Maryland).

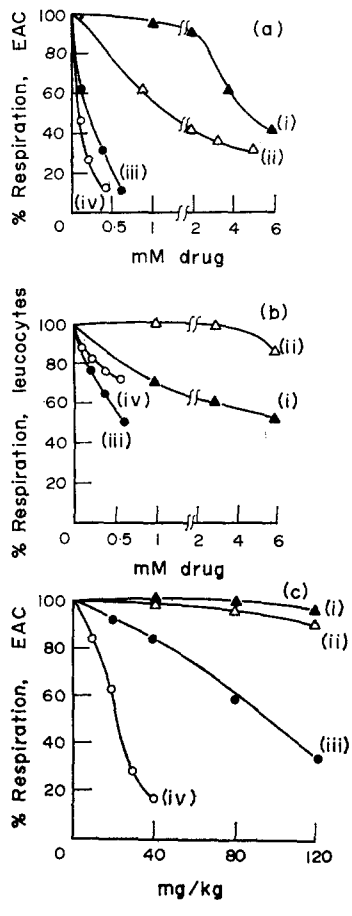


Fig. 4. (a) Effect of increasing concentrations of daunomycin (i), adriamycin (ii), ellipticene (iii) and VM 26 (iv) on respiration with endogenous substrate of a suspension of Ehrlich ascites tumor cells at 100 million cells per ml. (b) Effect of increasing concentrations of daunomycin (i), adriamycin (ii), ellipticene (iii) and VM 26 (iv) on respiration with endogenous substrate of a mixed population of pig blood leucocytes and lymphocytes, at 200 million cells per ml. One hundred per cent respiration in Fig. 4(a) and (b) was 120 n atoms of oxygen per min. (c) Respiration with endogenous substrate of 20 million cell samples of Ehrlich ascites cells, collected from 6 day grown Ehrlich ascites tumors, 4 hr after injection with increasing doses of daunomycin (i), adriamycin (ii), ellipticene (iii) and VM 26 (iv). One hundred per cent respiration was 24 n atoms/min.

contrary was observed in the case of adriamycin. Ellipticene, and to a greater extent, VM-26, showed much less effect on leucocyte respiration than on that of the tumor cells at the tested doses. From the shape of the inhibition curves with ellipticene and VM-26, it can be deduced that much larger concentrations of the drugs would be necessary to cause a total inhibition of leucocyte respiration than that needed for tumor cells.

Assuming that the use of similar oxygen utilization rates allows us to compare the drug concentrations required for inhibition in the 2 populations of cells, adriamycin, ellipticene and, above all, VM-26, appear to be respiratory inhibitors specific to varying degrees for tumor cells. In Fig. 4(c) are plotted the respiratory rates of Ehrlich ascites cells extracted from 6-day grown tumors treated 4 hr *in vivo* with a range of doses of the 4 drugs. It is shown that VM-26 at 40 mg/kg (130% of the chemotherapeutic dose employed for 1-day old tumors) inhibits 80% of the respiration of the cells. Ellipticene produces a 60% inhibition of respiration at twice the chemotherapeutic dose while daunomycin, at 3 times the chemotherapeutic dose level, has little effect on the respiration of the cells. It is significant that adriamycin, even at 28 times the chemotherapeutic dose level, shows no significant inhibition of respiration in the ascites tumor cell suspension. Taking into account the fact that the number of cells in a tumor grown for 6 days is much larger than the number of cells in a 1-day old tumor, and that the treatment of the cells with doses of ellipticene and VM-26 very close to the chemotherapeutic level resulted in a yield of very intensely inhibited cells, we may conclude that ellipticene, as has been shown for VM-26 [2] is a drug whose cancerostatic activity is possibly related to its action on cellular respiration.

DISCUSSION

The distinctive pattern of inhibition of NADH oxidation at low concentrations and of succinate oxidation at higher concentrations, with an accompanying uncoupling of oxidative phosphorylation, is familiar to those working with alkyl guanidino and barbiturate inhibitors [13]. However, the anticancer agents tested in these studies give mixed responses which are in part similar to those of the above-mentioned compounds but include others as well. Considering first adriamycin (Figs. 1 and 2): in State 4, while the respiration is only slightly stimulated, there is a clear oxidation of NADH suggesting interaction of adriamycin with the energy coupling mechanisms of Site I. At the same time, cytochrome *b* changes its steady state very little, suggesting a Site I interaction, mainly of an uncoupling nature. In State 3, however, we see clear inhibition of respiration (Fig. 1) accompanied by an oxidation of cytochrome *b* (Fig. 2) and eventually a reduction, as the adriamycin concentration is increased. Here we find some parallel to the thiopental effects [13] at the lower adriamycin concentrations while at the higher ones a cytochrome *b* reduction is observed. This pattern indicates a multi-site interaction of

adriamycin with the respiratory chain which is not observed with uncouplers and inhibitors of the types so far studied. It is not possible therefore to assign its action to a single site of the respiratory chain at this time and we identify therefore the response of the mitochondrial respiratory chain to such agents as relatively non-specific. While a portion of the effect, particularly that at low concentrations, may indeed involve specific binding sites at Site I and Site II, the total pattern indicates other effects as well. It is possible that, in addition to specific inhibition sites, membrane occupancy by the anticancer agents may contribute to their inhibitory effects.

The possibility that inhibitors of mitochondrial respiration and oxidative phosphorylation in general have anticancer activity is a promising one, and surely worthy of further investigation. However, the details of the proof of this hypothesis are difficult, since they involve the following considerations: relationships between the concentrations of drugs required for respiratory inhibition in isolated mitochondrial preparations and the anticancer concentrations required to produce the same effects *in vivo* may be complex, since such factors as drug distribution, drug metabolism and time-dependent binding phenomena may vastly alter the situation *in vivo* as compared with *in vitro*. Nevertheless, the levels of concentrations required for inhibition of respiration in isolated mitochondria provide initial estimates of the concentrations which would be required *in vivo* to produce comparable effects. For example, drugs acting at 1 μM concentrations, e.g. rotenone, would very probably have intolerably toxic effects [1]. Similarly, drugs such as ellipticene and VM-26, acting in the concentration range 25–100 μM, will probably have toxic effects or mechanisms of action related to their effect on respiration. Inhibition of respiration in mitochondria at 1 mM, or even slightly higher drug concentrations, may still have relevance to the *in vivo* treatment, depending on the factors of binding specificity, metabolism, time-dependency of action, and distribution, mentioned earlier, e.g. adriamycin.

The varying degrees of specificity of inhibition of tumor cell respiration described above for VM-26, ellipticene and adriamycin, are based on the comparison of the action of the drugs on cells with equivalent oxygen uptake. Finally, in order to ascertain the specificity of a respiratory inhibitor for tumor cells, it would be desirable to extend these studies to cover a wide range of tumor and normal cells, extracted after *in vivo* treatment with the relevant drug. It has been reported that several animal and human tumors have biochemical alterations of their NADH-linked respiratory components, or Site I of the respiratory chain [14, 15]. Although such data will possibly explain the difference in susceptibility of tumor cells to Site I inhibitors, further work along such lines is desirable.

Our results indicate that the effects of anticancer drugs on respiration are biologically significant properties of these agents, which can be helpful in understanding the results of biochemical screening tests, biochemical side effects of drugs, and, in some cases, provide a clue for new and significant mechanisms of action.

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REFERENCES

1. M. J. Figueras and M. Gosalvez, Inhibition of the growth of Ehrlich ascites tumors by treatment with the respirator inhibitor rotenone. *Europ. J. Cancer* **9,** 529 (1973).
2. M. Gosalvez, J. Perez-Garcia and M. Lopez, Inhibition of NADH linked respiration with the anticancer agent 4'demethyl-epipodophyllotoxin thenylidene glucoside. VM26. *Europ. J. Cancer* **8,** 471 (1972).
3. W. C. Schneider, Intracellular distributions of enzymes, III. The oxidation of octanoic acid by rat liver fractions. *J. biol. Chem.* **176,** 259 (1948).
4. B. Chance and B. Hagihara, Direct spectroscopic measurements of interactions of components of the respiratory chain with ATP, ADP phosphate and uncoupling agents. *Proc. Vth Inter. Cong. Biochem.* p. 3 Edn. Pergamon Press, Oxford (1961).
5. B. Chance and B. Hess, Metabolic Control Mechanisms. I. Electron transfers in the mammalian cell. *J. biol. Chem.* **234,** 2404 (1959).
6. B. Chance and G. R. Williams, Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. biol. Chem.* **217,** 409 (1955).
7. A. G. Gornall, C. J. Bardawill and M. N. David, Determinations of serum proteins by means of the Biuret reaction. *J. biol. Chem.* **177,** 751 (1949).

8. B. CHANCE and G. R. WILLIAMS, The respiratory chain and oxidative phosphorylation. *Advanc. Enzymol.* **17,** 65 (1956).
9. G. BONADONNA, S. MONFARDINI, M. DE LENA, F. FASSETI-BELANI and G. BERETTA, Phase I and preliminary phase II evaluation of adriamycin (NSC 123127) *Cancer Res.* **30,** 2572 (1970).
10. G. BONADONNA, G. MONFARDINI, S. DE LENA and F. FASSETI-BELANI, Clinical evaluation of adriamycin. A new antitumor antibiotic. *Brit. med. J.* **3,** 503 (1969).
11. G. BONADONNA, S. MONFARDINI and S. DIPIETRO, Clinical evaluation of adriamycin. *Int. Cancer Cong. Abst.* **10,** 478 (1970).
12. B. CHANCE, In preparation.
13. B. CHANCE and G. HOLLUNGER, Inhibition of electron and energy transfer in Mitochondria. *J. biol. Chem.* **238,** 418 (1963).
14. A. C. AISENBERG, *The Glycolysis and Respiration of Tumours* Academic Press, New York (1961).
15. T. OHNISHI, J. G. HEMINGTON, K. F. LA NOUE, H. P. MORRIS and J. R. WILLIAMSON, Electron paramagnetic resonance studies of iron sulfur centers in mitochondria prepared from three Morris hepatomas with different growth rates. Submitted to *Biochem. biophys Res. Comun.* (1973).

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Absence of Lymphomas in CBA Mice Derived by Embryo Transfer and Born from Lymphoma-prone AKR Mice

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Abstract—*Early embryo transfer has been used here to investigate susceptibility to lymphomas in mice. Lymphoma resistant CBA were transplanted at the early blastocyst stage, derived and milk fostered from lymphoma-prone AKR. In spite of this, the tumour resistance of the CBA was maintained. The fact that no lymphomas were noted suggests that neither the transplacental nor the transmammary route is of significance in determining lymphoma susceptibility in this situation. CBA would therefore appear to possess one or more genes at loci (other than H-2, Fv-1 and Fv-2 susceptibility loci) which render them resistant to lymphomas.*

INTRODUCTION

THREE different genetic loci have been linked with virus induced tumour susceptibility. One locus is the major histo-compatibility locus, H-2, where H-2^k is associated with tumour susceptibility. The other loci are Fv and designated Fv-1 and Fv-2. Here tumour susceptibility is associated with the alleles Fv-1^n and Fv-2^s (for review concerning these loci see Lilly and Pincus [1]). It is well known that the AKR are susceptible to Gross virus-associated lymphomas, and as might be expected, this strain is H-2^k, Fv-1^n and Fv-2^s. However, this is also true for the CBA, and in spite of this, they are considered in general to be resistant to lymphomas [2]. Gross [3] clearly demonstrated the role [2, 3] of the oncongenic virus in the AKR lymphoma. It is now generally assumed that this is normally acquired by vertical transmission which is possible either before or after implantation. Here we have investigated vertical transmission at and beyond the stage of implantation and resultant tumour susceptibility using the technique of early embryo transfer. CBA embryos were obtained and transplanted at the early blastocyst stage into pseudopregnant AKR recipients. Successfully derived progeny were milk fostered upon the same AKR recipients and subsequently investigated here for the development of lymphomas.

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MATERIAL AND METHODS

Mice

Conventionally housed inbred AKR and CBA/H-*T6T6* mice were used for this study. The AKR were originally obtained as AKR/J —maintained at the National Institute of Medical Research (NIMR), Mill Hill, for several years and for the last 4 years here at the Clinical Research Centre (CRC). CBA/H-*T6T6* were derived from the original Harwell stock, again introduced into NIMR, Mill Hill, before being bred at the CRC. The H-2^k status of both sublines have recently been confirmed (Dr. D. A. L. Davies, unpublished data).

Transplantation

CBA/H-*T6T6* (CBA in text) blastocysts were obtained after natural mating and transplanted into pseudopregnant AKR recipients.

The technique has been described in detail previously [4]. Successfully derived embryo transplanted CBA were subsequently milk fostered upon the same AKR recipient.

Examination

The exchange embryo transplantation derived CBA were examined clinically at weekly intervals, together with groups of AKR and CBA controls. In the event of declining health or any signs suggestive of a tumour (dyspneoa, marked lymphadenopathy or hepato-splenomegaly) the mice were then sacrificed and examined at post-mortem. Certain mice were examined at post-mortem after natural death and others at elective intervals in later life (1–2 years). In general, examination was macroscopic, although on occasions when there was any doubt, haematoxylin and eosin stained sections were prepared and examined histologically.

RESULTS

Controls

One hundred and sixty-three AKR controls were examined. Seven of these were either sacrificed in view of declining health (4 mice) or else were found dead (3 mice). Although the cause of ill health/death was generally uncertain, in no animal was a lymphoma found. The remaining 156 mice showed a 100% incidence of lymphoma between the ages of 16–56 weeks. Certain of these data have been presented previously [5] and now are summarised in Fig. 1.

Seventy-three CBA controls were examined. As can be seen from Fig. 2, the majority of these survived up and until elective sacrifice late in life. It can also be seen from Fig. 2 that certain mice died naturally or were sacrified in view of declining health. In all three groups tumours were found. These were generally hepatomas, the exception being two aged males with well differentiated adenocarcinoma of the lung. Marked hydronephrosis was a feature of three mice, all males, sacrificed between 72–88 weeks of age.

Embryo transplanted CBA (H-2T6T6)

One hundred and eighty-four CBA blastocysts were transplanted into pseudopregnant AKR recipients. Regretfully only 63 survived beyond weaning. The commonest cause of foetal loss appeared to be cannibalism in the immediate postnatal period. The 63 (42♂, 21♀) CBA survivors were derived from 11 of 21 AKR recipients.

With the exception of 8 mice, all these embryo transplanted CBA lived and were well up and until elective sacrifice at 68–89 weeks of age. The exception were 3 mice that were found

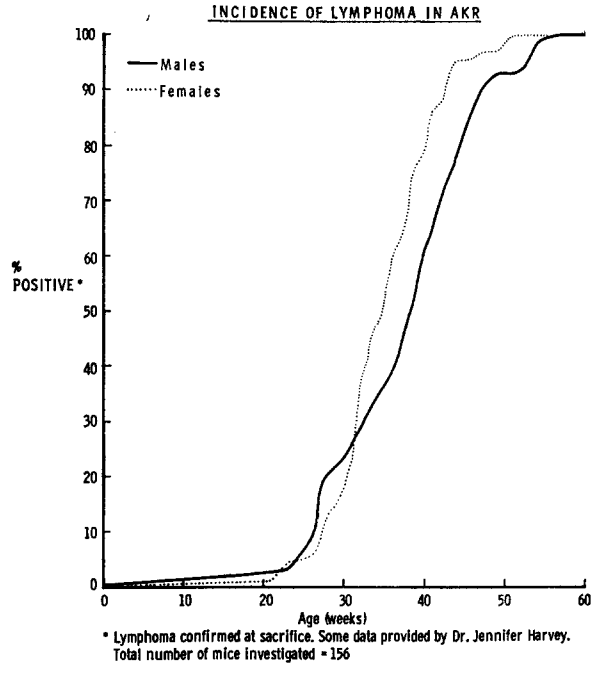


Fig. 1. Incidence of lymphoma in normally derived AKR mice.

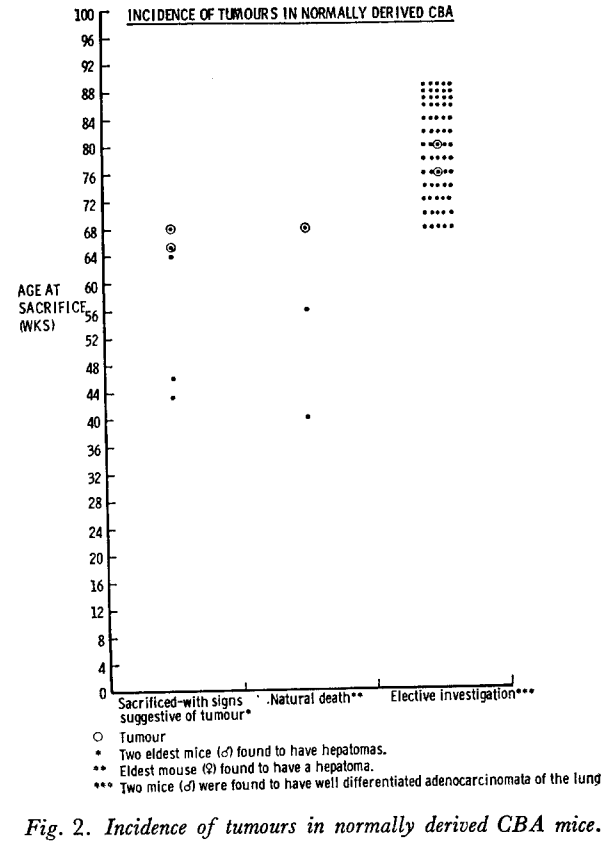


Fig. 2. Incidence of tumours in normally derived CBA mice.

dead. One of these was noted to have a hepatoma, but in the other two the cause of death could not be determined. In spite of this, there was no evidence of lymphomas in any of these mice, or in the 5 sacrificed with signs suggestive of a tumour. Two of these 5 mice were found to have hepatomas. Two of the mice sacrificed between 68–89 weeks without any abnormal clinical signs, were later found to have tumours. One male of 80 weeks was seen to have a hepatoma, whilst the other, a female of 76 weeks of age, was noted to have a well differentiated adenocarcinoma of the lung (Fig. 3).

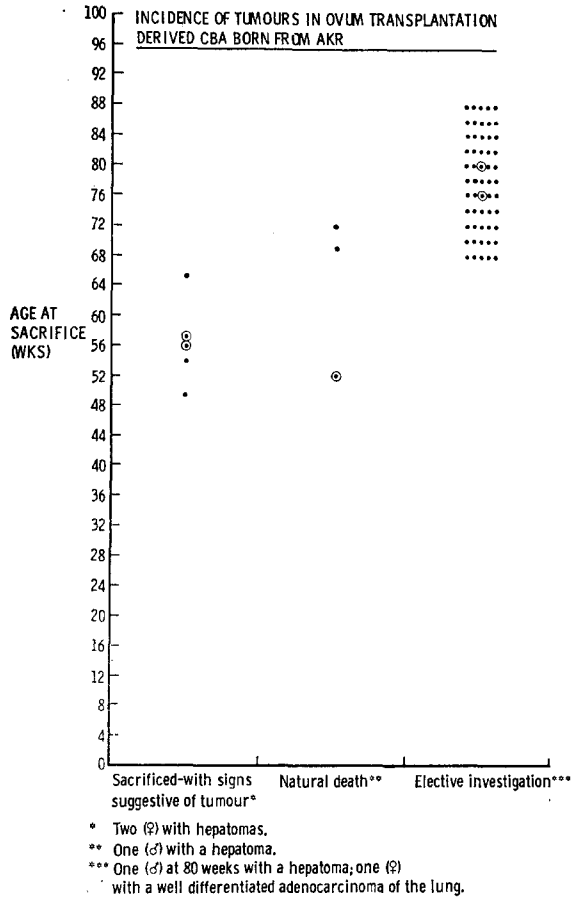


Fig. 3. Incidence of tumours in ovum transplantation derived CBA mice born from AKR.

DISCUSSION

Results clearly confirm lymphoma susceptibility of the AKR. Recently it has been stressed that the overall incidence varies between sublines in the AKR [6]. In our colony the incidence of lymphomas is 100% and is reached by 56 weeks of age [5]. In contrast, lymphomas were not seen in any of the CBA controls. Tumours were occasionally observed in these CBA but these were generally hepatomas. The tumour resistance of the CBA [2] is confirmed, and furthermore, this is not altered in spite of being ovum transplantation derived and milk fostered from the lymphoma-prone AKR.

The fact that normally derived CBA do not develop virus-associated lymphomas is itself interesting since, like the AKR, it is H-2^k. Fv-1^n and also Fv-2^s positive. In spite of this it remains tumour resistant even after being uterine nurtured from the AKR. In this situation, vertical transmission is possible at or beyond the stage of implantation. Although it has yet to be shown that these transplantation derived CBA have acquired the oncogenic AKR virus, if this were the case, then this would be further evidence of a genetic factor in the CBA that could overcome the oncogenic susceptibility associated with the H-2^k, Fv-1^n and Fv-2^s positive loci. Earlier evidence of this effect was obtained in a group of ovum fusion derived tetraparental AKR↔CBA chimaeras. In spite of being predominantly AKR (peripheral blood > 95%) [7], lymphomas were rare in these chimaeras [5]. This in turn has suggested that relatively few CBA cells have in some way contributed to tumour resistance. How this has been achieved remains to be determined. Conceivably the oncogenomic expression of the virus might be altered in some way and thus tumours do not develop. Alternatively tumours may occur but are successfully rejected [8]. Either way evidence points to a third genetically determined, and furthermore, dominant factor, present in the CBA that enables it to overcome the innate susceptibility of the AKR. Independent of the mechanism there is already evidence for this since as mentioned earlier, the CBA, like the AKR, are also H-2^k, Fv-1^n and Fv-2^s positive. These three factors are associated with virus-associated tumour susceptibility. In spite of this the CBA are tumour resistant [2] and furthermore, this is unaltered in spite of being uterine nurtured from the AKR. The genetically determined dominant factor associated with tumour resistance in the CBA remains to be identified.

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REFERENCES

1. F. Lilly and T. Pincus, Genetic control of murine viral leukaemogenesis. *Advanc. Cancer Res.* **17,** 231 (1973).

2. E. D. MURPHY, Characteristic tumours. In *Biology of the Laboratory Mouse*, 2nd Edn., (Edited by E. L. GREEN), p. 521, Blakiston Division/McGraw-Hill New York (1966).
3. L. GROSS, "Spontaneous" leukemia developing in C3H mice following inoculation, in infancy, with AK-leukemic extracts, or AK-embryos. *Proc. Soc. exp. Biol. (N.Y.)* **76,** 27 (1951).
4. R. D. BARNES, M. TUFFREY, J. KINGMAN and R. A. RISDON, The disease of the NZB mouse. Examination of exchange ovum transplantation dervied NZB and CFW mice. *Clin. exp. Immunol.* **10,** 493 (1972).
5. R. D. BARNES, M. TUFFREY and C. E. FORD, Suppression of lymphoma development in tetraparental AKR mouse chimaeras derived from ovum fusion. *Nature New Biol.* **244,** 282 (1973).
6. R. T. ACTON, E. P. BLAKENHORN, T. C. DOUGLAS, R. D. OWEN, J. HILGERS, H. A. HOFFMAN and E. A. BOYSE, AKR mice—genetic variation among sublines. *Nature New Biol.* **245,** 8 (1973).
7. M. TUFFREY, R. D. BARNES, E. P. EVANS and C. E. FORD, Dominance of AKR lymphocytes in tetraparental AKR↔CBA-T6T6 chimaeras. *Nature New Biol.* **243,** 207 (1973).
8. R. D. BARNES, Leukaemia—an immune deficiency? *Advanc. Biosci.* **12,** (In press) (1974).

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Growth of Human Leukaemic Peripheral Blood Cells in Diffusion Chambers

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Abstract—*In these investigations, the diffusion chamber technique was used to characterise the growth of peripheral blood cells from 10 patients with acute leukaemia and, for comparison, from 4 normal persons. By means of the Isopaque–Ficoll method, erythrocytes and granulocytes were removed and the resulting mononuclear cell suspension cultured for various times up to 3 weeks in diffusion chambers implanted into pre-irradiated CBA mice.*

The growth pattern for cells from normal persons was uniform with an initial decrease in total cell number between 3 and 6 days after implantation and a maximum at day 13, due mainly to the appearance of blast cells, granulopoietic cells and macrophages, with few erythropoietic precursors and megakaryocytes. In the leukaemic patients, the growth patterns differed widely from normal and also between the patients themselves. Five patients had an almost continuous cell increase exceeding normal, largely due to leukaemic blast cells and granulopoietic cells, whereas other patients had growth curves similar to normal or a continuous decrease in cell number over the whole period. The extent of cell growth might be related to the diagnosis, since patients with marked increase in cell number had acute myeloid leukaemia or acute erythroleukaemia whereas patients with practically no growth at all had acute undifferentiated leukaemia or acute lymphoid leukaemia.

In some patients the number of granulopoietic cells in diffusion chambers greatly exceeded the corresponding number from normal cells and it is assumed that some maturation of leukaemic blast cells took place. From the high number of leukaemic blast cells which developed in the diffusion chambers in spite of a low ^{3}H-thymidine labelling index in peripheral blood, it is concluded that either a small population of blast cells was capable of continued self-replication or that some non-proliferating "quiescent" leukaemic cells re-entered proliferation.

INTRODUCTION

RECENT ideas on the pathogenesis of human acute leukaemia favour the possibility that the haematological stem cells are involved in the leukaemic process [1, 2]. Any attempt to provide a functional characterization of acute leukaemia should include, therefore, the possibility of testing for stem cell function. Up to now, tests for the pluripotent stem cell have been confined to animal experiments, and for humans only the granulopoietic committed stem cell could be assayed using the *in vitro* colony technique. Now, the diffusion chamber culture system [3, 4], which has been shown to provide a suitable milieu for growth of the murine pluripotent stem cell [5–7], offers a method to test for all three committed stem cells, if not for the pluripotent stem cell, in human material. Evidence for this comes from the development of immature cells of the granulopoietic and erythropoietic series and megakaryocytes in diffusion chambers following the implantation of peripheral blood or bone marrow cells [8–10].

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In the present study, the diffusion chamber technique has been used to investigate the growth of peripheral blood leukocytes from 10 patients with varying forms of acute leukaemia, and to determine the proliferative capacity and possible maturation of leukaemic blast cells. For comparison, cells from the peripheral blood of four normal persons were studied in the same way.

MATERIAL AND METHODS

Clinical details and laboratory findings for the 10 patients studied are summarized in Table 1. The patients were investigated before treatment was started. The diagnoses of acute myeloid leukaemia (AML), acute erythroleukaemia (AEL), acute undifferentiated leukaemia (AUL) and acute lymphoid leukaemia (ALL) given in the table were based on morphological and cytochemical observations of bone marrow smears. In case 7, the undifferentiated leukaemia was atypical, with appearance of granulopoietic precursors in the peripheral blood later in the disease (see Table 4). In 2 patients, (cases 2 and 3) the diagnosis of acute erythroleukaemia was based on the erythroid hyperplasia of the bone marrow, whereas in the peripheral blood there were mainly peroxidase positive blast cells and only some erythropoietic precursors. The details of differential blood counts for the patients are given in Table 4.

Table 1. *Clinical data and peripheral blood counts for* 10 *patients studied with acute leukaemia*

Patient	Age	Sex	Diagnosis	Cytochemistry Peroxidase	Esterase	PAS	Leukocytes (cells/μl)	Leukaemic blast cells (cells/μl)	Erythrocytes (10^6/μl)	Platelets (cells/μl)
1. P.G.	31	M	AML	+	–	–	45,200	24,600	1·58	11,000
2. H.J.	27	F	AEL	+	–	–	7000	2600	2·70	33,000
3. H.E.	17	F	AEL	+	–	–	2800	600	3·30	103,000
4. B.G.	53	M	AML	+	–	–	167,000	124,900	3·36	78,000
5. H.T.	49	F	AML	+	–	(+)	5700	1700	2·59	21,000
6. H.F.	56	M	AML	+	–	–	4000	600	1·86	45,000
7. A.W.	17	M	AUL first relapse	–	–	(+)	10,700	700	2·32	39,000
8. S.A.	23	F	AUL first relapse	–	–	–	38,700	33,300	3·35	120,000
9. B.J.	59	F	ALL	–	–	+	19,500	2000	3·30	241,000
10. T.A.	18	F	AUL first relapse	–	–	–	29,000	19,200	4·10	188,000

Preparation of cell suspension

Mononuclear cells were separated by removal of erythrocytes and granulocytes using the Isopaque–Ficoll method of Bøyum [11]. Peripheral blood samples (20–40 ml) were taken from the patients with EDTA as anticoagulant (15 ml blood + 3 ml 1·107% Na_2EDTA in 0·7% NaCl) and diluted with 3 parts of 0·9% NaCl solution. Volumes of 16 ml diluted blood were carefully layered on to 6 ml of a mixture of 10 parts 33·9% Ronpacon* and 24 parts 9% Ficoll† solution in tubes of internal diameter 25 mm. After centrifugation at 20°C for 40 min at 400 *g*, the supernatant was discarded and the interface layer of mononuclear cells collected with a Pasteur pipette and pooled. These cells were washed twice with phosphate-buffered saline and resuspended in TC199 medium buffered to pH 7·2 [3] and containing 500 units penicillin G and 50 μg streptomycin per ml. The cell concentration was adjusted to 5×10^6 cells/ml. All glassware was siliconized.

From 2 patients (P.G. and H.J.), these separated cells were stored under liquid nitrogen and thawed just before implantation.

^{3}H-thymidine labelling

Peripheral blood was incubated for 1 hr with 2 μCi/ml of ^{3}H-thymidine (specific activity 20 Ci/mM) at room temperature. Autoradiographs of smears were prepared with Ilford G5 liquid emulsion and exposed for 20 days for determination of the labelling index.

Diffusion chambers

The diffusion chamber technique [12] was carried out according to the description by Benestad [13], Bøyum and Borgstrøm [3] and Breivik

*Ronpacon(R)—Cilag Chemie, GmbH, Alsbach

†Deutsche Pharmacia GmbH, Frankfurt/M.

[5]. The chambers were constructed by glueing to each side of a Millipore plexiglas ring* (10 mm inner dia, 2 mm thick) MF-Millipore filters of pore size 0·22 μm with Millipore MF cement. Before use, the chambers were tested for leaks and sterilized by dry heat at 80°C for 24 hr.

Filling procedure

Chambers were filled with 100 μl of cell suspension (5×10^5 cells) using a tuberculin syringe and 20 gauge needle. The filling orifice was then closed with a short length of nylon rod and sealed with Tensol Cement No. 6†. The cement was allowed to dry and the chambers stored in ice-cold buffered TC 199 medium until implantation.

Implantation

CBA mice, pre-irradiated with 650 R (250 kVp, 1 mm Cu filter, 2·6 mm Cu H.V.L., 80 R/min), were anaesthetized with ether and 2 chambers per host mouse inserted in the abdominal cavity through a midline incision. About 0·5 ml TC 199 medium containing antibiotics was instilled and the wound closed with clips. After 7 and 14 days, chambers were removed, cleaned and reimplanted into fresh pre-irradiated CBA mice.

Harvesting

Chambers were usually harvested at 1, 3, 6, 9, 13, 17 days after implantation and at 21 days if sufficient cells were available. At each time, 6 chambers were removed, cleaned and shaken in vials containing 3 ml of a 0·5% pronase‡-TC 199 medium for 1 hr at room temperature to give a single cell suspension [14]. For determination of the total nucleated cells in individual chambers the following procedure of Bøyum *et al.* [9] was used. The filter membrane was pierced with a fine Pasteur pipette and the chamber contents transferred to a small preweighed conical tube. The chamber was rinsed 3 times with buffered EDTA solution (9 parts buffered saline, 1 part 2·7% EDTA) and the washings added to the chamber contents. The total volume was found by weighing and the nucleated cell concentration determined in a haemocytometer so that the total nucleated cells could be calculated. Finally, the tubes were centrifuged (5 min at 200 *g*), the cell pellet resuspended in 20% albumin solution and smears prepared. The cells were stained with May Grünwald–Giemsa or prepared for autoradiography or cytochemistry.

Evaluation

Differential counts of nucleated cells were carried out as follows: for peripheral blood leukocytes, 200 cells on each of 2 smears and for the cell suspensions used for implantation, 500 cells on each of 2 smears were counted. For the differentiation of the cells growing in diffusion chambers, counts were made for the day of maximum cell numbers (day 13) in normal persons and the same time chosen also for the leukaemic patients; 500 cells for each of 4 chambers harvested at this time were counted.

Granulopoietic cells were divided into 8 stages of maturation, M_1–M_8, according to the description of Cronkite and Fliedner [15], where M_1 = myeloblast, M_2 = promyelocyte, M_3 = immature myelocyte, M_4 = mature myelocyte, M_5 = metamyelocyte, M_6 = juvenile, M_7 = band form and M_8 = segmented form. Large, medium and small lymphocytes were counted together as one population.

The ^{3}H-thymidine labelling index in autoradiographs was determined on 1000 blast cells.

RESULTS

The effect of the cell separation procedure using the Isopaque–Ficoll method on the peripheral blood of normal persons and of patients with acute leukaemia is shown in Table 2 which compares the differential counts on peripheral blood and on the cell suspension after separation. In addition to erythrocytes, granulocytes are removed so that for normal persons the remaining constituents are mainly lymphocytes and monocytes. In the leukaemic patients, some metamyelocytes (M_5) and juveniles (M_6) remain, but the main constituents of the chamber inoculum are leukaemic blast cells and lymphocytes, and in Case 7 (A.W.) also granulopoietic cells, M_2–M_4.

Growth from normal cells

The change in the total nucleated cells per chamber with time after implantation of 5×10^5 mononuclear cells is illustrated in Fig. 1. In each of the 4 normal persons studied, the pattern was similar, consisting of an initial decrease of about 10–35% with a minimum between 3 and 6 days of culture, followed by an increase in cell number to a maximum on the 13th day, after which the total cell number again decreased.

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†ICI, Welwyn Garden City.
‡Pronase P reinst. Serva, Heidelberg.

The types of cells developing in diffusion chambers (summarized for the 4 normal persons as the mean and range) are given in Table 3, where the chamber contents at the time of maximum cell number (day 13) are compared with the chamber inoculum. Besides an increase in the lymphocytes in all 4 normal persons, new populations of cells appeared which were not present in the implanted inoculum, most impressive of these being the blast cells, which ranged in number from 87,000 to 150,500 cells/chamber. Of these blast cells some were lymphoid in appearance but their indefinite morphology did not allow them to be allotted to a particular cell series. Immature and fewer granulopoietic precursors developed in numbers ranging from 5000 to 13,700 and 2200 to 9900 cells respectively. They were morphologically somewhat abnormal but identifiable by having a positive peroxidase reaction. Macrophages, including a few monocytes, comprised 7500–57,000 cells. Megakaryocytes were present in all chambers; erythropoietic precursors were rare and not listed in the table.

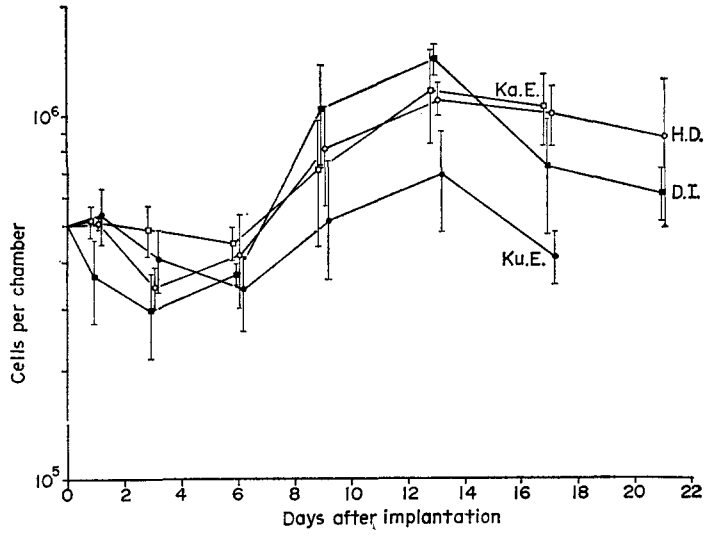


Fig. 1. Change in total number of nucleated cells per chamber with time after implantation of 5×10^5 *mononuclear peripheral blood cells from normal persons. Chambers were implanted into pre-irradiated hosts* (650 *R*) *and re-implanted after* 7 *and* 14 *days into new pre-irradiated hosts. Mean values of* 4–6 *chambers at each time of harvesting are given together with their standard deviation.*

Growth from leukaemic cells

The changes in total cell number per chamber for the leukaemic patients are shown in Fig. 2 as individual curves which have been arranged as far as possible in order of the extent of growth. The first 5 patients (P.G., H.J., H.E., B.G. and H.T.) formed a group in which the initial decrease was brief and after a minimum on the first day after implantation, the cell number increased more or less continuously so that either the "maximum" occured at the last time of harvesting or the curve showed only a slight decrease (patients B.G. and H.T.), later than the normal decrease after 13 days. In these 5 patients, the total cell number increased well above the implanted inoculum. The last 5 patients were not so uniform but, in general, were characterized by the fact that the total cell number per chamber either merely regained the implanted cell number or remained below. Two of these patients, (H.F. and S.A.), showed a pattern somewhat similar to normal, with an initial decrease at 3–6 days and a suggestion of a maximum at 13 days. One patient (A.S.) was distinguished by an extensive initial decrease. In the last 2 patients (B.J. and T.A.) the total cell number decreased over the whole period. Considering this total cell growth in relation to the patients' diagnoses, it is noticeable that the patients with a large increase in total cell number have the diagnosis AML or AEL, whereas poor growth is associated with the diagnosis of AUL or ALL.

Maturative and proliferative capacities of leukaemic cells

From the comparison in Table 3 of the differential counts of the implanted cell suspension and of the chamber contents at day 13, it is obvious that the marked increase in total cell number in the first group of patients was

Table 2. Differential counts of leukocytes in peripheral blood from 10 leukaemic patients before and after cell separation using the Isopaque–Ficoll method

			Peripheral blood (%)					Cell suspension (%)				
						Granulopoietic cells					Granulopoietic cells	
	Patient	Diagnosis	Blast cells	Lymphocytes	Monocytes	M_2–M_4	M_5–M_8	Blast cells	Lymphocytes	Monocytes	M_2–M_4	M_5–M_8
1.	P. G.	AML	54·5	31·5	1·5	1·5	10·3	76·3	19·1	1·7	0·5	1·7
2.	H. J.	AEL	37·0	37·0	—	—	4·0	65·3	9·5	0·5	0·1	2·9
3.	H. E.	AEL	22·0	33·0	4·0	—	37·0	47·2	41·0	9·4	0·2	0·9
4.	B. G.	AML	74·8	4·5	0·8	2·5	17·2	91·6	3·9	1·1	0·6	2·3
5.	H. T.	AML	29·0	37·5	—	0·5	31·0	51·3	47·0	0·7	0·1	0·1
6.	H. F.	AML	14·8	58·0	0·5	—	25·7	12·1	79·6	0·7	0·8	6·0
7.	A. W.	AUL	6·4	7·0	1·6	6·2	78·6	20·5	36·5	7·5	25·5	10·0
8.	S. A.	AUL	86·0	4·5	—	0·7	8·8	90·3	7·1	1·0	1·0	0·6
9.	B. J.	ALL	10·5	64·0	1·0	—	24·5	8·0	83·4	6·0	0·2	2·4
10.	T. A.	AUL	66·2	10·2	1·6	—	22·0	84·4	10·0	4·4	—	1·2
	Mean for 4 normal persons		—	38·4	3·8	—	57·8	—	80·8	17·5	—	1·0
	Range			33·0–42·0	2·0–6·5		51·0–63·7		73·8–92·4	6·3–24·8		0–2·7

Table 3. Chamber content at the time of implantation and after 13 days culture of cells from 10 leukaemic patients

Patient	Diagnosis	Chamber inoculum: Total cells $\times 10^5$	Blast cells	Lymphocytes	Monocytes	Granulopoietic cells M_2–M_4	Granulopoietic cells M_5–M_8	Chamber contents at day 13: Total cells* $\times 10^5$	Blast cells*	Lymphocytes*	Granulopoietic* cells M_2–M_4	Granulopoietic* cells M_5–M_8	Macrophages*	Day 17 Megakaryocytes
1. P. G.	AML	5·50	419,700	105,100	9,400	2,800	9,400	10·33	833,000	12,000	22,000	36,700	39,100	+
2. H. J.	AEL	4·73	308,500	44,900	2,400	500	13,700	11·83	814,000	42,100	145,000	54,000	7300	−
3. H. E.	AEL	5.03	237,000	206,000	47,200	1000	4500	14·91	845,000	162,300	134,700	15,800	32,700	+
4. B. G.	AML	4·93	451,100	19,200	5400	3000	11,300	10·52	976,000	7900	8400	13,000	27,800	−
5. H. T.	AML	4·80	246,200	225,600	3400	500	500	12·80	339,600	396,700	90,700	164,600	73,600	−
6. H. F.	AML	4·50	54,500	358,200	3200	3600	27,000	5·40	161,200	414,100	9100	4400	7000	−
7. A. W.	AUL	3·70	75,900	135,100	27,800	94,400	37,000	5·25	108,100	215,200	115,400	50,400	24,100	−
8. S. A.	AUL	4·50	406,400	32,000	4500	4500	2700	3·38	308,600	18,000	6900	1200	200	+
9. B. J.	ALL	5·03	40,200	419,500	30,200	1000	12,100	2·08	1800	190,800	1500	600	11,600	+
10. T. A.	AUL	4·98	419,000	49,800	21,900	—	6000	1·64	179,400	13,900	700	200	2200	+
Mean for 4 normal persons		4·94	—	403,500	83,300	—	3,400	11·00	112,900	896,300	9,500	6,400	32,300	+
Range		4·50 to 5·30		332,100 to 474,200	18,800 to 131,400		1,000 to 8,600	6·92 to 14·31	87,000 to 150,500	438,500 to 1,264,100	5,000 to 13,700	2,200 to 9,900	7,500 to 56,900	

*Mean values from 4 to 6 chambers.

due largely to the growth of leukaemic blast cells. In contrast to growth from normal cells, the leukaemic patients had a decrease in the number of lymphocytes, with the exception of cases 6 and 7 (H.F. and A.W.). The number of macrophages which arose was in or near the normal range. A difference from normal is seen in the megakaryocytes, which were not always found during the differential count of 2000 cells.

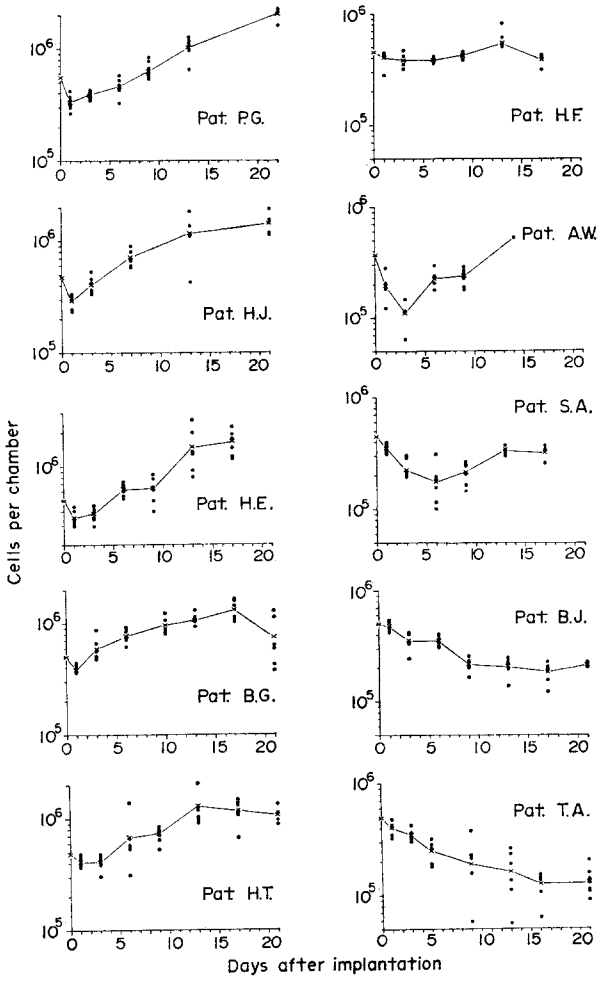


Fig. 2. Change in total number of cells per chamber with time of implantation of 5×10^5 *mononuclear cells separated from the peripheral blood of* 10 *patients with varying forms of acute leukaemia. Each point represents the value from a single chamber and the lines join the mean values at each time of harvesting.*

The number of granulopoietic precursors at day 13 exceeded the normal range in 5 patients (P.G., H.J., H.E., H.T., and A.W.). Here, as in the growth from normal persons, the granulopoietic cells were mostly immature, morphologically somewhat abnormal, but identified by being peroxidase positive. These granulopoietic precursors theoretically could arise from the granulopoietic cells implanted, from remaining normal stem cells included in the lymphocyte population, or could be differentiated descendents of the leukaemic blast cells. There was no relation between the number of granulopoietic cells with the lymphocytes implanted (see Fig. 3). Since it is therefore unlikely that all the granulopoietic precursors were descendants from normal stem cells included in the lymphocyte population, the possibility of maturation of the leukaemic blast cells to granulopoietic precursors is favoured.

It was of further interest to see how far the growth of leukaemic blast cells in diffusion chambers was correlated to the fraction of proliferating leukaemic blast cells in the peripheral blood, as determined by their labelling index

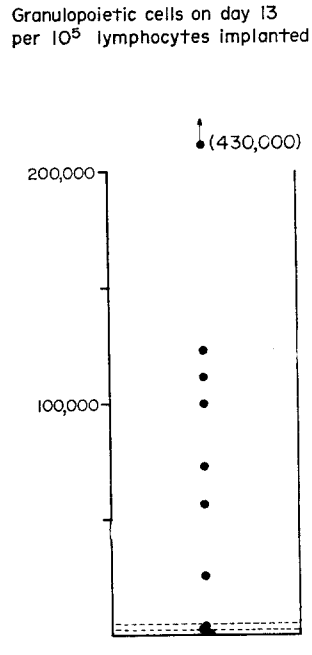


Fig. 3. Yield of granulopoietic precursor cells in diffusion chambers after 13 *days of culture, normalised to* 10^5 *lymphocytes implanted. The dotted lines indicate the range of corresponding values in normal persons.*

with ^{3}H-thymidine. Of the 6 patients for whom the labelling index was determined, the patient A.W., with an atypical form of acute leukaemia, had an unusually high labelling index, for which no reason was apparent. Neither for these labelling indices nor for the number of implanted blast cells calculated to be in the S-phase from the labelling indices, could a correlation be found with the number of leukaemic blast cells at day 13 ($P > 0{\cdot}05$, see Table 4).

DISCUSSION

For the diffusion chamber culture of cells from leukaemic patients, peripheral blood was chosen rather than bone marrow because of

its easier availability and also because it might have significance in being an exchange organ between various sites of leukaemic cell proliferation. The blood was separated by the Isopaque–Ficoll method to remove granulocytes, which are present in varying proportions in the blood of leukaemic patients, and thus eliminate possible growth inhibition by chalones produced from these cells [16]. The use of a mononuclear cell suspension for implantation also has the advantage that the subsequent appearance of immature precursor cells can be taken as evidence of their development in the chamber and interpretation is not complicated by the possibility that these cells are survivors from precursors implanted, as is the case for bone marrow [10], although this advantage is somewhat limited in leukaemic patients where not all the granulopoietic precursors were removed.

In the investigations described here, host mice were pre-irradiated in order to increase stimulation and to minimize possible immunological effects on the growth of human cells [9]. Under the experimental conditions of pre-irradiation, times of re-implantation and times of harvesting used, a pattern of growth from normal cells was observed with an initial decrease of 10–35%; thereafter, proliferation of blast cells, lymphocytes, granulopoietic cells and macrophages led to a maximum at day 13. This growth pattern is similar to that for the culture of human bone marrow cells in diffusion chambers observed by Bøyum *et al.* [9] and Boecker *et al.* [8]. Megakaryocytes and rare erythropoietic precursors were seen, and, since only lymphocytes and monocytes were implanted, the appearance of these precursors is good evidence that even if the human pluripotent stem cell does not proliferate in diffusion chambers then at least all 3 committed stem cells do so.

The growth of cells from leukaemic patients showed marked differences from the normal pattern and also between the patients themselves. In the first 5 patients, an early increase in number was found, starting after the first day, followed by a more or less continuous cell growth above the normal maximum and it seems likely that this is due to the proliferation of leukaemic blast cells. As indicated by the differential counts on the chamber contents at day 13, the increase in cell number is due mainly to the appearance of leukaemic blast cells and granulopoietic precursors. In contrast to normal cell growth, there is a decrease in the number of lymphocytes. In the 2 patients (H.F. and S.A.), where the pattern of total chamber cellularity was similar to normal, the initial decrease and rise of cell numbers to a maximum at day 13 might be an indication of the development of a normal cell population from normal haemopoietic stem cells in the implanted cell suspension, unless the leukaemic cells in these patients have a proliferative behaviour similar to normal. A maximum at day 13 from residual normal cells was probably not observable in the first 5 patients because of masking by the overwhelming growth of leukaemic blast cells. It should be noted that the patient in this group (H.F.) with AML, a diagnosis associated with marked cell proliferation in the first 5 patients, had a low content of leukaemic blast cells in the implanted inoculum. In the last 2 patients there was apparently no appreciable cell proliferation, either of leukaemic blast cells or of normal cells. The latter could be either because the remaining normal stem cells were too few or because normal cell growth was suppressed by the leukaemic cells in these patients.

The peripheral blood cells from leukaemic patients thus showed a wide variation in their growth patterns and the question arises as to

Table 4. Relationship between ^{3}H-thymidine labelling index (LI) of leukaemic blast cells in peripheral blood and growth of blast cells in diffusion chambers

Patient	L.I. of leukaemic blast cells in blood $\times 10^5$	Blast cells per chamber: Total number implanted	Blast cells per chamber: Number in S-phase calculated from L.I.	Blast cells per chamber: Total number at day 13
1. P. G.	9·4	419,700	39,500	833,000
2. H. J.	0·3	308,500	930	814,500
3. H. E.	1·4	237,000	3320	845,000
7. A. W.	86·0	75,900	65,300	63,000
8. S. A.	1·1	406,400	4500	308,600
10. T. A.	1·5	419,900	6300	179,400

what this variation might be attributed.

Considering the patients' diagnoses in relation to the total cell growth, or more particularly to the number of leukeamic blast cells at day 13, it appears that marked growth of leukaemic blast cells was associated with patients having AML or AEL, whereas poor growth was associated with patients having AUL or ALL. Since diffusion chambers provide a favourable environment for the development of granulopoiesis from normal haemopoietic stem cells, it could be that blast cells from the patients with AML find a suitable milieu for their proliferation and that the diffusion chamber milieu does not promote the growth of undifferentiated or lymphoid blast cells. In support of this, Chikkappa *et al.* [17] found good growth of bone marrow cells from 2 patients with chronic myelocytic leukaemia. However, poor growth in diffusion chambers was found with cells from 2 patients with AML by Laerum *et al.* [18], although these results are not strictly comparable since several factors in their experimental conditions were different (e.g. rats used as hosts and not pre-irradiated). From the few patients studied here it might be assumed that the growth from peripheral blood cells from leukaemic patients is related to their diagnosis, but the contrary findings of Laerum *et al.* [18] indicate that this might not necessarily be the case.

When the growth patterns of these patients are considered in relation to the course of their disease (the response to therapy and survival time) there appears to be also an association, in so far as marked cell growth was found in patients with a short survival time and vice versa [19]. However, this association should be viewed with caution since treatment of the patients was not uniform. If there are individual growth patterns for the leukaemic patients this might lead to a functional characterization with indications for prognosis but clearly more patients have to be studied.

It is of interest to investigate whether the diffusion chamber culture provides information on the ability of leukaemic blast cells to mature. Although no direct qualitative tests were undertaken (e.g. chromosome analysis), some indirect quantitative indications are given by the number of immature granulopoietic cells found in chambers at 13 days, which in 5 cases were in excess of normal values. In 4 of these patients P.G., H.J., H.E. and H.T.), it is probable that the majority of these cells did not come from granulopoietic precursors in the inoculum, since these were too few (Table 3). Similarly, it is unlikely that the granulopoietic cells are descendants from normal haemopoietic stem cells in the implanted lymphocytes, unless it is assumed that the concentration of stem cells within the lymphocyte population is increased. There is thus an indication that some granulopoietic cells found in chambers must be matured descendants from the leukaemic blast cells. Such maturation is apparently abortive since the granulopoietic cells were mostly immature precursors and had morphological alterations. These findings are in agreement with other suggestions that leukaemic blast cells can mature [20, 21]. Since megakaryocytes and erythropoietic precursors appeared in normal or reduced numbers, it seems most likely that these cells are derived from normal stem cells (although maturation from leukaemic blast cells cannot be excluded) and that in cases where such cells were not found, normal stem cells were too few or normal cell growth was suppressed by the leukaemic cells.

In acute leukaemia, the low labelling index of blast cells in the peripheral blood is usually taken as an indication that the majority of these cells are non-proliferating. However, they are not necessarily all end-cells but may be partly "quiescent" cells in a long G_1-phase or in G_0-phase capable of re-entry into proliferation, as discussed in detail by Killmann [22]. In the patients investigated here, the leukaemic blast cells showed also a low ^{3}H-thymidine labelling index, with the exception of patient A.W. In spite of is, in that least 3 patients, (P.G., H.J. and H.E., Table 4), the growth of leukaemic blast cells in diffusion chambers was much greater than would have been expected from the labelling index. Two explanations can be put forward for the high numbers of cells produced. One is that a small fraction of proliferating cells may be capable of several divisions. Here, even under the assumptions that no blast cells die in the chambers, that the normal number of blast cells develop from remaining normal stem cells, and that a greater proportion of cells are actually in cycle than are measured to be in the S-phase by the ^{3}H-thymidine labelling index, about 3 divisions in patient P.G. and 8 divisions in patient H.J. would be required to achieve the numbers of blast cells observed at day 13. This would imply that within the leukaemic blast cells, a small population exists which is capable of continued self-replication and thereby has "stem-cell" properties. Alternatively, there may be quiescent cells which re-enter into proliferation under the stimulus of diffusion chamber culture. Whichever of these explanations is valid, and whether or not

it may have significance *in vivo*, the culture of leukaemic blast cells in the diffusion chamber system seems to give insight into their potential for proliferation.

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REFERENCES

1. S. A. Killmann, Acute leukaemia: development, remission/relapse pattern, relationship between normal and leukaemic hemopoiesis, and the "sleeper-to-feeder" stem cell hypothesis. *Ser. Haematol.* **1,** 103 (1968).
2. E. A. McCulloch and J. E. Till, Leukaemia considered as defective differentiation: complementary *in vivo* and culture methods applied to the clinical problem. In *The Nature of Leukaemia.* (Edited by P. C. Vincent) p. 119. Blight, Govt. Printer, Sydney, (1972).
3. A. Bøyum and Borgstrøm, The concentration of granulocytic stem cells in mouse bone marrow, determined with the diffusion chamber technique. *Scand. J. Haemat.* **7,** 294 (1970).
4. H. Breivik, H. B. Benestad and A. Bøyum, Diffusion chamber and spleen colony assay of murine haemopoietic stem cells. *J. cell. Physiol.* **78,** 65 (1971).
5. H. Breivik, Haemopoietic stem cell content of murine bone marrow, spleen and blood. Limiting dilution analysis of diffusion chamber cultures. *J. cell. Physiol.* **1,** 73 (1971).
6. A. Bøyum, A. L. Carsten, O. D. Laerum and E. P. Cronkite, Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers. Effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. *Blood* **40,** 174 (1972).
7. W. S. Tyler, E. Niskanen, F. Stohlman, J. Keane and D. Howard, The effect of neutropenia on myeloid growth and the stem cell in an *in vivo* culture system. *Blood* **40,** 634 (1972).
8. W. R. Boecker, A. Bøyum, A. L. Carsten and E. P. Cronkite, Human bone marrow (HBM) and blood (HPB) stem cell kinetics. *Blood* **38,** 819 (1971).
9. A. Bøyum, W. Boecker, A. L. Carsten and E. P. Cronkite, Proliferation of human bone marrow cells in diffusion chambers implanted into normal or irradiated mice. *Blood* **40,** 163 (1972).
10. E. P. Cronkite, A. L. Carsten, G. Chikkappa, J. A. Laissue and S. Öhl, Culture of normal and leukemic cells in diffusion chambers. In *Prognostic Factors in Human Acute Leukaemia.* (Edited by T. M. Fliedner and S. Perry) Pergamon Press, (In press).
11. A. Bøyum, Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest. Suppl.* **97,** 77 (1968).
12. G. Algire, J. M. Weaver and R. T. Prehn, Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in diffusion chambers in mice. *J. nat. Cancer Inst.* **15,** 493 (1959).
13. H. B. Benestad, Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. *Scand. J. Haemat.* **7,** 279 (1970).
14. P. Nettesheim, T. Makinodan and C. J. Chadwick, Improved diffusion chamber cultures for cytokinetic analysis of antibody response. *Immunology* **11,** 427 (1968).
15. E. P. Cronkite and T. M. Fliedner, Granulocytopoiesis. *New Engl. J. Med.* **270,** 1347 and 1403 (1967).
16. T. Rytömaa and K. Kiviniemi, Control of granulocyte production I. Chalone and antichalone, two specific humoral regulators. *Cell Tiss. Kinet.* **1,** 329 (1968).
17. G. Chikkappa, W. R. Boecker, A. L. Carsten, E. P. Cronkite and S. Öhl, Return of leukocyte alkaline phosphatase in chronic myelocytic leukemia marrow cells cultured in a diffusion chamber system. *J. clin. Invest.* **52,** 18a (1973).

18. O. D. LAERUM, A. GRÜNEISEN and M. F. RAJEWSKY, Proliferative properties of malignant cell populations cultured in intraperitoneal diffusion chambers. *Europ. J. Cancer* **9,** 533 (1973).
19. D. HOELZER, E. B. HARRISS and E. KURRLE, Prognostic indications of diffusion chamber and agar culture studies in human acute leukemia. In *Prognostic Factors in Human Acute Leukemia.* (Edited by T. M. FLIEDNER and S. PERRY) Pergamon Press, Oxford, (In press).
20. S. A. KILLMANN, A hypothesis concerning the relationship between normal and leukaemic haemopoiesis in acute myeloid leukaemia. In *Hemopoietic Cellular Proliferation* (Edited by F. STOHLMAN) p. 267. Grune & Stratton, New York 1970.
21. W. A. ROBINSON, J. E. KURNICK and B. L. PIKE, Colony growth of human leukemic peripheral blood cells *in vitro. Blood* **38,** 500 (1971).
22. S. A. KILLMANN, Kinetics of leukaemic blast cells in man. *Clinics in Haemat.* **1,** 95 (1972).

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Age at First Birth: An Equivocal Factor in Human Mammary Carcinogenesis

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Abstract—*Age at clinical onset of mammary cancer in relation to parity and age at first birth was studied in 790 patients. One would expect to find a retardative effect of high parity and early delivery since these factors were found to be protective as far as mammary cancer risk is concerned. The conclusions of these investigations are in complete opposition to this original hypothesis: in the present series, breast cancer appears to occur significantly earlier in women with a high parity and whose first delivery occurred early. Thus, the same process appears to be protective as far as total mammary cancer risk is concerned, but accelerating as far as age at tumor onset is concerned. The different possibilities of bias were discussed; they do not seem to account for this unexpected finding.*

INTRODUCTION

RECENT interest has centered on the relationship between genito-obstetrical status and breast cancer risk. Three variables in particular have been investigated: age at menarche, age at menopause and pregnancies. In most reports it was found that early menarche and late menopause are statistically related to an increase of this risk. As for pregnancies, the reverse association between the number of children a woman has borne and her risk of developing the disease has been known for a long time. But recently, knowledge of this relationship was refined when MacMahon and his group demonstrated that the protective effect is actually correlated to the age of the mother at the first birth rather than to the total number of children; thus, a woman first parous before the age of 18 has about one-third the breast cancer risk of a woman whose first delivery is delayed until the age of 35 [1, 2]. These two obstetrical conclusions are by no means inconsistent, since mothers with a high parity usually have their first child early.

A study was undertaken in the Centre Francois Baclesse on the hypothesis that these factors—age at menarche, age at menopause, parity or age at first birth—may also be related to the age of the woman at clinical onset of the mammary malignancy. Expected findings were that breast cancer appears earlier in the case of early menarche, late menopause, low parity or late first delivery, since there is evidence of the unfavourable significance of these factors as far as cancer risk is concerned.

The first results of these investigations were reported in two preliminary papers [3, 4]. The starting hypothesis proved to be correct as far as the influence of early menarche and late menopause on the age at cancer onset is concerned (though not significantly for the late menopause). However, in opposition to this hypothesis, it was found that breast cancer appears earlier in multiparous women and in women who had an early first parturition. Thus, multiparity and early first birth, considered as protective factors as far as breast cancer risk is concerned, appear to be accelerating factors when the age at clinical cancer onset is taken into account.

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Owing to the paradoxical feature of this conclusion, a complementary investigation seemed to be justified.

MATERIAL AND METHODS

The source of this study consists of the records of 869 female patients treated for carcinoma of the breast at the Centre Francois Baclesse from August 1970, to August 1973. Of this number, 79 cases were excluded as not being reliable because of the condition of the patient: cachexy, senility, mental deficiency, deafness, language difficulties, etc. On the whole, it was possible to ascertain parity and age at first birth in 790 patients.

Parity was defined in terms of deliveries, whether liveborn or still-born. In the study of the age at first birth, abortions were excluded but still-born children included.

For each patient it was admitted that the age at clinical onset of the disease was that at which the first therapeutic procedure, whether surgical, radiational, chemical or hormonal, took place. This convention obviously involves a possibility of error as not all patients take medical advice within the same time-lapse. The possible consequences of this fact will be discussed later.

RESULTS

A. *The relationship between parity and the age at clinical onset of mammary cancer*

A total of 790 women were included in this study. The results are given in Table 1.

Table 1. *Age at mammary cancer onset in relation to total parity in* 790 *patients* (*nulliparous:* 237; *parous:* 553)

Number of births	Number of patients	Mean age at breast cancer onset (yr)
0	237	58·00
1	143	58·90
2	143	53·73
3	122	53·67
4	63	54·87
5	35	58·91
6 and more	47	56·89

As a confirmation of our preliminary paper, it was found that breast cancer appears earlier after a high than after a low parity. This conclusion is hardly statistically significant when one compares nulliparous with parous ($0{\cdot}03 < P < 0{\cdot}04$), but definitely significant when one compares pauciparous (nulliparous +1 para) with multiparous ($P < 0{\cdot}001$).

B. *The relationship between the age at first birth and the age at clinical onset of mammary cancer*

This study concerns 553 patients whose age at first birth could be ascertained. The results are shown in Fig. 1.

Thus, here again, one comes to a conclusion which is a confirmation of our preliminary study and in contradiction to the original hypothesis. In our series, breast cancer occurs significantly earlier in patients who bore their first child at an early age ($P < 0{\cdot}001$); proportionally, the later the first delivery, the later the onset of the disease.

DISCUSSION

In the case of total parity, attention should be drawn to the particularity of para 1 women where the clinical onset of cancer occurred on average later (58·90 years) than in nulliparous (58·00 years). This finding is contrary to that of any of the categories of multiparous women. An explanation of this peculiarity is found when one considers the average age of the patients at the time of their first delivery in relation to their total parity (Table 2). It can be seen that para 1 women had their delivery at the average age of 28·06. On the other hand, Fig. 1 demonstrates that when one considers the age at cancer onset in relation to the age at first birth, nulliparous women are placed on the same footing as women first parous at 29 years, therefore, about the same age as para 1. This being so, one can understand our findings as shown in Table 1, where the differences are hardly significant when nulliparous are compared with parous, but highly significant when pauciparous (para 0 and 1) are compared with multiparous (para 2 and more).

The evidence of breast cancer onset occurring earlier in women who bore their first child at an early age leads to consideration of possible bias.

Firstly, one might ask whether the convention according to which the age at tumor onset and the age at which first treatment occurs are identified is valid. Several investigators have pointed out that older patients usually delay longer than younger patients prior to taking medical advice on their mammary lesion. However, the difference in these delays is only a matter of months [5]. It cannot, therefore, account for the important difference found in the present investigation, i.e. about 15 years

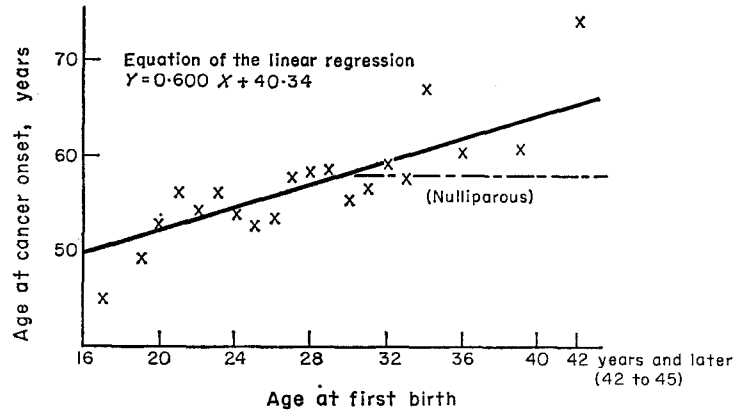


Fig. 1. *Average age at breast cancer onset in relation to age at first birth in* 553 *women.*

Table 2. *Age at first birth in relation to total parity in* 553 *patients*

Number of births	Number of patients	Mean age at first birth (yr)
1	143	28·06
2	143	25·34
3	122	25·05
4	63	24·35
5	35	23·23
6	15	22·27
7	13	23·15
8 and more	19	23·00

between the average age at mammary cancer onset in females first parous before 20 years of age and those first parous at 32 years of age or later (Fig. 1).

A second source of possible bias relates to the fact that women who develop a breast cancer at an early age obviously cannot have had a late pre-cancerous first pregnancy. It therefore might be asked whether the results expressed in Fig. 1 are not a mere consequence of this evidence rather than an actual retarding effect of a late pregnancy. In order to make the force of this objection clear, this study was resumed after exclusion of all women who developed a breast cancer prior to 45 years of age (123 cases), so that the obstetrical possibilities have been identical in all the remaining patients (430 cases). Under these conditions, it was again found that breast cancer occurs earlier in patients who bore their first child at an early age. However, the equation of the linear regression is $Y = 0{\cdot}264\ X + 53{\cdot}60$ instead of $Y = 0{\cdot}600\ X + 40{\cdot}34$, with a fall of the statistical significance from $P < 0{\cdot}001$ to $0{\cdot}01 < P < 0{\cdot}02$. (Fig. 2).

Another objection could be raised due to the fact that women currently have their first child earlier, on the average, than they did 30 or 40 years ago. This argument cannot be retained for the same reason as the first objection, because this trend towards earlier first pregnancy in the younger generations appears to range in France about only 1–2 years, if one refers to published data. [6]

One of the main problems set by these investigations consists of the connection of their results with the results of those conducted by MacMahon's group. As was previously

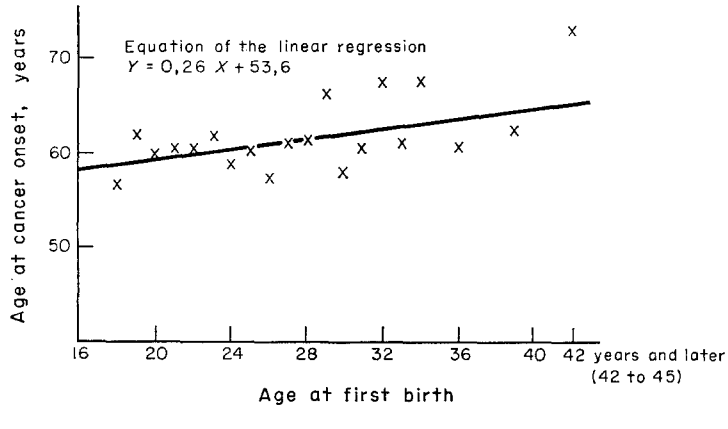


Fig. 2. *Average age at breast cancer onset in relation to age at first birth in* 430 *women whose cancer appeared after* 45 *years of age.*

mentioned, the present study was undertaken with the expectation of coming to a conclusion which seemed to be consistent with the investigations of this group, i.e. later breast cancer following earlier pregnancy. In fact, it has led to the exact opposite. On the other hand, it is striking to see that in both investigations, nulliparous women roughly have the same equivalence on the scale of age at first birth: 30 years of age in the papers of MacMahon's group and 29 years of age in the present study. It is unlikely that this similarity results from mere chance. It appears more satisfactory to hypothesize that both studies express the same biological process which, in the case of early delivery, inhibits the mechanism of mammary carcinogenic induction and therefore reduces the total number of cancers, but which subsequently quickens the evolution of the inducted disease. Such a conjecture implies that breast cancers should be more severe following an early first birth. Strictly speaking this topic does not seem to have given rise to investigations up to now. MacKay and Sellers, however, reported that the 5-year survival rate of patients operated on for breast cancer was lower in women who had had 5 or more deliveries (42·8%) than in those of lower parity (51–52%), which is an indirect corroboration of this hypothesis [5]. A more direct corroboration results from the study of 114 patients of the Centre Francois Baclesse who were operated on and found to have 3 or more axillary nodes involved. The 3-year survival rate was 42·1% in 38 patients who had first given birth prior to 24 years of age, and 53·9% in 76 patients who were nulliparous or who had first given birth at 24 years of age or later.

To summarize, it may be said that after an early first birth, whereas the total risk of breast cancer decreases, the onset of the disease appears to be earlier. This apparent paradox may be accounted for by a faster growth of the malignancy.

REFERENCES

1. B. MacMahon, P. Cole, T. M. Lin, C. R. Lowe, A. P. Mirra, B. Ravnihar, E. J. Salber, V. G. Valaoras and S. Yusa, Age at first birth and breast cancer risk. *Bull. Wld Hlth Org.* **43,** 209 (1970).
2. B. MacMahon and P. Cole, Etiology of human breast cancer: a review. *J. nat. Cancer Inst.* **50,** 21 (1973).
3. P. Juret, J. Robillard, J. E. Couette, D. Brune and J. C. Vernhes, La carcinogenèse mammaire: le rôle équivoque de la grossesse. *Nouv. Presse méd.* **2,** 2363 (1973).
4. P. Juret, J. Robillard, J. E. Couette, D. Brune and J. C. Vernhes, Données épidémiologiques apparemment contradictoires en matière de cancer du sein. *Sem. Hôp. Paris (Sem. méd. prof. méd-soc.)* **50,** 43 (1974).
5. E. N. MacKay and A. H. Sellers, Breast cancer at the Ontario Cancer Clinics, 1938–1956: a statistical review. *Can. med. Ass. J.* **92,** 647 (1965).
6. Demographic Yearbook, Publishing Service of United Nations, p. 284 (1950), p. 406 and 480 (1965) and p. 343 and 387 (1969).

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6-Methyl-8-β-Ergoline-Acetonitrile (MEA)-Induced Suppression of Mammary Tumorigenesis in C3H/HeJ Female Mice*

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Abstract—*Daily treatment for* 14 *months of* 2*-month-old nulliparous C3H/HeJ mice with 6-methyl-8-β-ergoline-acetonitrile (MEA), an effective inhibitor of pituitary prolactin secretion, markedly suppressed development of mammary hyperplastic alveolar nodules and sharply reduced mammary tumor incidence. Mature* 8*-month-old multiparous C3H/HeJ mice, treated daily for* 30*-day periods, at bimonthly intervals for a period of* 1 *year, also had reduced numbers of mammary hyperplastic alveolar nodules and decreased mammary tumor incidence. Treatment with the ergoline derivative had no significant effect on pituitary, ovarian, uterine, adrenal or body weight nor did it alter the estrous cycles. Thus, significant inhibition of spontaneous mammary tumorigenesis by drug-induced hormone (prolactin) suppression has been demonstrated in this study.*

INTRODUCTION

REZABEK *et al.*, [1] reported that acute admistration of 6-methyl-8-β-ergoline-acetonitrile (MEA) to rats reduced the incidence of and, under certain conditions, totally prevented pregnancy in these animals. Mantle and Finn subsequently confirmed this anti-fecundity activity (inhibition of ova implantation) of the ergoline derivative in mice [2]. These authors suggested that the action of the drug was primarily due to suppression of pituitary prolactin secretion, an action comparable to the chemically similar but more thoroughly investigated ergot alkaloids [3–5]. The prolactin-suppression activities of MEA have been confirmed in rodents by radioimmunoassay [6, 7].

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It is widely recognized that prolactin is an important hormone in murine mammary tumorigenesis [8–13]. Treatments which increase the secretion of this hormone markedly enhance the development and growth of either spontaneous [8, 9, 14] or carcinogen-induced [10, 15] mouse or rat mammary tumors. Recently, Welsch and Clemens [16] reported that acute or chronic treatment of female mice with MEA significantly reduced the incidence and development of mammary hyperplastic alveolar nodules, hyperplasias which are believed to antedate the carcinoma [17]. It was of interest, therefore, to determine whether or not mammary tumor-susceptible mice, if treated chronically with MEA, would respond with a convincing reduction of mammary carcinoma incidence parallel to that of the reduction of these pre-neoplastic growths.

MATERIAL AND METHODS

All animals used in this study were C3H/HeJ female mice obtained from the Jackson

Laboratories, Bar Harbor, Me. They were housed in a temperature—(24±1°C) and light-controlled (14 hr/day) room and provided a diet of Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) and water *ad libitum*.

Treatment of young nulliparous mice for 14 months with MEA

One hundred 2-month-old mice were injected s.c. daily for 14 months with 0·1 mg MEA suspended in saline. The MEA suspension (1 mg/ml) was prepared by dissolving the drug initially in a minimal amount of ethanol and diluting to volume with saline. Ethanol constituted 1·0% or less of the final suspension. A second group of 90 2-month-old mice, injected s.c. daily with the saline–ethanol solution, served as controls. Estrous cycles were determined in the MEA-treated and control mice approximately 8 months after the onset of treatment.

Eight months after cessation of treatment (24 months of age), the surviving mice were sacrificed. Pituitaries, ovaries and uteri were excised and weighed. Inguinal mammary glands were excised, spread flat on cork, fixed in 15% formalin and stained for wholemount evaluation by a standard procedure. Mammary glands were rated for development according to the following criteria: (1) few ducts, few or no end buds; (2) moderate duct growth, moderate number of end buds; (3) numerous ducts and branches, many end buds; (4) numerous ducts and branches, minimum lobulo-alveolar growth; (5) numerous ducts and branches, moderate lobulo-alveolar growth; and (6) numerous ducts and branches, dense lobulo-alveolar growth as in late pregnancy. The number of hyperplastic alveolar nodules (HAN) were counted in the wholemount preparation. Only hyperplastic nodular outgrowths equal to or greater than 0·5 mm in dia were recorded for computation. Wholemount preparations were examined under 10-fold magnification and coded prior to grading.

All mice were weighed weekly and examined for mammary tumors at monthly intervals from the onset of treatment. Mean difference between organ weight and number of HAN were evaluated statistically by Student's *t*-test. Mean differences between mammary gland development were evaluated statistically by the non-parametric Wilcoxon rank procedure test. Differences between mammary tumor incidence were evaluated by Chi-square analysis.

Treatment of mature multiparous mice for one year with MEA

Seventy 8-month-old mice (retired breeders), free of palpable mammary tumors, were injected s.c. daily with 0·1 mg MEA for 30-day periods, at bimonthly intervals, for one year. Seventy 8 month-old mice, similarly treated, but with the diluent only, served as controls. All mice were weighed weekly and examined for mammary tumors at monthly intervals from the onset of treatment. At 19 months of age and 24 hr after the last 30-day treatment period, all surviving mice were sacrificed. Pituitaries, uteri, ovaries and adrenals were excised and weighed. Inguinal mammary glands were excised and evaluated for development and number of HAN as described previously.

RESULTS

Treatment of young nulliparous mice for 14 months with MEA: effect on hyperplastic and tumorous mammae development

Young nulliparous mice were treated daily with MEA for 14 months (from 2 to 16 months of age). The mice received no treatment from 16 to 24 months of age, after which time they were sacrificed. MEA treatment significantly: (1) reduced number of mammary hyperplastic

Table 1. Effects of treatment (14 months) of young C3H/HeJ nulliparous mice with MEA on degree of mammary gland development, number of mammary hyperplastic nodules and mammary tumor incidence

	Treatment*	
	Controls	MEA
Number of mice at beginning of study	90	100
Number of mice at 24 months of age†	16	11
Mean inguinal mammary gland development at 24 months of age	3·8	3·4
Mean number of hyperplastic nodules in inguinal mammary glands at 24 months of age	14·6 ± 1·7[a]	5·6 ± 1·0[b]
Number of mice which developed mammary tumors	24/90(27%)[a]	8/100(8%)[b]

*All mice were treated daily for 14 months beginning at age 2 months, terminating at 16 months of age. No treatment from 16 months to 24 months of age. Dose of MEA: 0·1 mg/mouse per day; controls were treated with saline.

†Number of surviving mice at end of study.

$P < 0·001$, a/b.

alveolar nodules (at 24 months of age) (Table 1) and (2) reduced the incidence of mammary tumors (2–24 months of age) (Table 1). At 24 months of age, 8 months after cessation of drug treatment, no significant effects of the treatment on body weight, pituitary, uterine or ovarian weight, or on mammary gland development were observed (Table 2). No apparent adverse effect on the general health of the mice was noted, either during or subsequent to the treatment.

Table 2. Effects of treatment (14 months) of young C3H/HeJ nulliparous *mice with MEA on organ weights*

	Treatment*	
	Controls	MEA
Number of mice at beginning of study	90	100
Number of mice at 24 months of age†	16	11
Mean body wt (g) at 24 months of age	29·8	31·6
Mean pituitary wt (mg) at 24 months of age	3·1 ± 0·2	2·9 ± 1·4
Mean uterine wt (mg) at 24 months of age	389·6 ± 73·7	479·9 ± 85·2
Mean ovarian wt (mg) at 24 months of age	19·6 ± 2·4	14·5 ± 2·2

*All mice were treated daily for 14 months beginning at age 2 months, terminating at 16 months of age. No treatment from 16 months to 24 months of age. Dose of MEA: 0·1 mg/mouse per day; controls were treated with saline. Means are accompanied by standard error.

†Number of surviving mice at end of study.

Mammary tumor incidence in the MEA-treated mice (8/100, 8%) was less than one-third that observed in the controls (24/90, 27%) Three of the mammary tumors in the MEA-treated group occurred during the last 3 months of the treatment period (14–16 months of age), and the other 5 occurred at 21–22 months of age, 5–6 months after cessation of drug treatment. Rate of death among controls and MEA-treated mice (nulliparous and multiparous) were similar, therefore, number of mice at risk in each group throughout this study were comparable. Mean number of hyperplastic alveolar nodules found in the inguinal mammary glands of the MEA-treated mice (5·6 ± 1·0) was nearly one-third that found in the controls (14·6 ± 1·7) 8 months after cessation of treatment (24 months of age). No effect of MEA-treatment on the length of the estrous cycle was observed.

Treatment of mature multiparous mice for one year with MEA: effect on normal, hyperplastic and tumorous mammae development

Daily treatment of mature multiparous mice with MEA for 30-day periods at bimonthly intervals for 1 year (from 8 months to 19 months of age) significantly: (1) suppressed mammary gland development (Table 3); (2) inhibited hyperplastic alveolar nodular formation (Table 3); and (3) reduced the incidence of mammary tumors (Table 3). No significant

Table 3. Effects of treatment (1 year) of mature C3H/HeJ multiparous *mice with MEA on degree of mammary gland development, number of mammary hyperplastic alveolar nodules and mammary tumor incidence*

	Treatment*	
	Controls	MEA
Number of mice at beginning of study	70	70
Number of mice at 19 months of age†	13	21
Mean inguinal mammary gland development at 19 months of age	3·5[a]	2·0[b]
Mean number of hyperplastic alveolar nodules in inguinal mammary glands at 19 months of age	15·9 ± 1·5[a]	7·9 ± 1·0[b]
Number of mice which developed mammary tumors	36/70(51%)[c]	23/70(33%)[d]

*All mice were treated daily, for 30 days, at bimonthly intervals for a period of 1 year (6 months total treatment). The treatments began when the mice were 8 months of age and ended when the mice were 19 months old. All mice were sacrificed 24 hr after the last 30-day treatment period. Dose of MEA: 0·1 mg/mouse per day; controls were treated with saline. Means are accompanied by standard error.

†Number of mice surviving at the end of the treatment period.

$P < 0·001$, a/b.

$P < 0·005$, c/d.

effect of the treatment on body weight, pituitary, uterine, ovarian or adrenal weight was observed when the mice were sacrificed (at 19 months of age), nor was there any apparent adverse effect of the treatment on the general health of the animals.

Thirty-six (51%) of the controls developed mammary tumors in contrast to 23 (33%) of the MEA-treated mice. Mean number of hyperplastic alveolar nodules observed in the inguinal mammary glands of the MEA-treated mice (7·9 ± 1·0) was approximately one-half that observed in the controls (15·9 ±

1·5). The mammary glands of the MEA-treated mice were considerably more atrophic than the controls. The control mice had mammary glands which were characterized by numerous ducts and end buds and some alveolar development, while the drug-treated mice had only moderate amounts of ductal and end bud development.

Table 4. *Effect of treatment* (1 *year*) *of mature C3H/HeJ* multiparous *mice with MEA on body and organ weights*

	Treatment*	
	Controls	MEA
Number of mice at beginning of study	70	70
Number of mice at 19 months of age†	13	21
Mean body wt (g) at 19 months of age	30·4	27·2
Mean pituitary wt (mg) at 19 months of age	2·8 ± 0·1	2·3 ± 0·1
Mean uterine wt (mg) at 19 months of age	238·4 ± 23·5	191·9 ± 13·1
Mean ovarian wt (mg) at 19 months of age	13·2 ± 1·7	10·6 ± 0·8
Mean adrenal wt (mg) at 19 months of age	5·9 ± 0·2	6·3 ± 0·2

*All mice were treated daily, for 30 days, at bimonthly intervals for a period of 1 year (6 months total treatment). The treatments began when the mice were 8 months of age and ended when the mice were 19 months old. All mice were sacrificed 24 hr after the last 30-day treatment period. Dose of MEA: 0·1 mg/mouse per day; controls were treated with saline. Means are accompanied by standard error.

†Number of mice surviving at the end of the treatment period.

DISCUSSION

Chronic treatment with MEA of either nulliparous or multiparous C3H/HeJ mice significantly reduced the incidence of hyperplastic as well as neoplastic mammary development. Treatment with the ergoline derivative did not impair body weight gains, alter the estrous cycles or adversely influence the general health of the animals.

MEA is an effective suppressor of prolactin secretion in mice and rats [2, 6, 7, 16]. The ergoline derivative reduces radioimmunoassayable prolactin in rats to near levels found in hypophysectomized animals [6, 7]. In estrogen-treated rats, whose prolactin secretion is markedly elevated [18], concurrent treatment of these animals with MEA also significantly reduces the serum levels of this hormone [7]. The drug appears to be relatively specific for the hormone, i.e. it does not appear to influence *markedly* other endocrine processes [6, 16]. Mice or rats treated with the drug experience normal estrous cycles, as reported previously [16] and also in this study.

Prolactin is acknowledged to be an important hormone in the genesis of mouse and rat mammary tumors [8–15]. Treatments which stimulate hypersecretion of this hormone, e.g. pituitary grafts [8, 14] hypothalamic lesions [15, 19, 20] or administration of tranquilizing drugs [9, 21], cause a marked increase in mammary tumor development in these animals. The results of the present study provide evidence that a reduction of *normal* secretory rates of the hormone significantly suppresses this neoplastic change while not interfering with normal ovarian activity. Mammary tumor incidence, as well as the number of hyperplastic alveolar nodules (HAN), in MEA-treated young nulliparous mice was approximately one-third that in the controls. In MEA-treated mature multiparous mice, mammary tumor incidence and HAN number were also significantly reduced, although this difference was not as striking as in the nulliparous mice. HAN are already numerous in mature multiparous mice which in all probability explains this disparity. Prophylaxis of spontaneous mammary tumorigenesis in rodents by drug (2-Br-α-ergocryptine) (CB-154)-induced suppression of pituitary prolactin secretion was recently reported by Welsch and Gribler [22]. The ergot (CB-154) appears to be a more effective inhibitor of mammary tumorigenesis in mice than in MEA at the same dose level, as more striking differences were observed between controls and experimentals in the ergot study [22] than in the present study. However, the ergot (CB-154) appears to be somewhat less tolerated by the mice than MEA, an obviously important consideration when determining the pharmacological acceptability of the two drugs. It is conceivable that the dose of MEA could be substantially increased, without significant toxicity, resulting in suppression of mammary tumor development equal to or exceeding that observed with CB-154. Interestingly, at least in rats, MEA is virtually equivalent to CB-154 in its effectiveness in reducing serum prolactin [6, 7].

The results of this study provide further evidence that prolactin is an important, perhaps essential, hormone in the *genesis* of spontaneous mouse mammary tumors. This hormone also appears to be important for the development of carcinogen-induced mammary tumors in the rat [23] and pituitary isograft-

induced mammary tumors in the mouse [24]. It is conceivable that limited availability of prolactin during the time period in which the conversion or progression to hyperplasia–neoplasia would ordinarily occur either (1) renders existing mammary cells metabolically refractory to the hyperplastic–neoplastic process or (2) interferes with the development of progenitive cells, whose progeny would be susceptible to hyperplastic–neoplastic transformation.

The role of prolactin in human mammary tumorigenesis is uncertain at present, although recent studies have provided evidence that this hormone may play a role in this process [25–29]. Advanced metastatic human breast cancers are, more often than not, hormone independent [30]. In the mouse, the vast majority of advanced mammary tumors are also hormone independent, although the early developmental stages of the disease are generally very responsive to the hormonal environment [16, 17, 22]. If prolactin can be shown to be important in the *development* of normal, hyperplastic or neoplastic mammae in man, as it is in rodents, prophylaxis of the disease may be possible by appropriate drug-mediated hormone suppression.

REFERENCES

1. K. Rezabek, M. Semonsky and N. Kucharczyk. Suppression of conception with D-6-methyl-8-cyanomethylergoline (1) in rats. *Nature (Lond.)* **221,** 666 (1969).
2. P. G. Mantle and C. A. Finn, Investigations on the mode of action of D-6-methyl-8-cyanomethylergoline in suppressing pregnancy in the mouse. *J. Reprod. Fertil.* **24,** 441 (1971).
3. M. C. Shelesnyak, Disturbance of hormone balance in the female rat by single injection of ergotoxin ethanesulphonate. *Amer. J. Physiol.* **180,** 47 (1955).
4. E. Flückiger, Drugs and the control of prolactin secretion. In *Prolactin and Carcinogenesis.* (Edited by A. R. Boyns and K. Griffiths), p. 162, Alpha Omega Alpha, Cardiff, Wales (1972).
5. C. W. Welsch, M. D. Squiers, E. Cassell, C. L. Chen and J. Meites, Median eminence lesions and serum prolactin: influence of ovariectomy and ergocornine. *Amer. J. Physiol.* **221,** 1714 (1971).
6. J. A. Clemens, C. J. Shaar, E. B. Smalstig, N. J. Bach and E. C. Kornfeld, Inhibition of prolactin secretion by ergolines. *Endocrinology* (In press).
7. C. L. Brooks and C. W. Welsch, Reduction of serum prolactin in rats by 2 ergot alkaloids and 2 ergoline derivatives: a comparison. *Proc. Soc. exp. Biol. (Med.)* (In press).
8. O. Mühlbock and L. M. Boot, Induction of mammary cancer in mice without the mammary tumor agent by isografts of hypophysis. *Cancer Res.* **19,** 402 (1959).
9. A. Lacassagne, J. F. Duplan, Le méconisme de la cancerisation de la mamelle chez la souris, considéré d'après les resultats d'expériences au mogen de la reserpine. *C. R. Acad. Sci. (Paris)* **249,** 810 (1959).
10. N. Haran-Ghera, The role of mammotrophin in mammary tumor induction in mice. *Cancer Res.* **21,** 790 (1961).
11. J. C. Heuson, C. Waelbroech-VanGaver and N. Legros, Growth inhibition of rat mammary carcinoma and endocrine changes produced by 2-Br-α-ergocryptine, a suppressor of lactation and nidation. *Europ. J. Cancer* **6,** 353 (1970).
12. C. W. Welsch and E. M. Rivera, Differential effects of estrogen and prolactin on DNA synthesis in organ cultures of DMBA-induced rat mammary carcinoma. *Proc. Soc. exp. Biol. (Med.)* **139,** 623 (1972).
13. J. Furth, Hormones and neoplasia. In *Cancer and Aging,* Thule International Symposia, p. 1. Nordiska Bokandelns Forlag, Stockholm (1968).
14. C. W. Welsch, T. W. Jenkins and J. Meites, Increased incidence of mammary tumors in the female rat grafted with multiple pituitaries. *Cancer Res.* **30,** 1024 (1970).
15. C. W. Welsch, J. A. Clemens and J. Meites, Effects of hypothalamic and amygdaloid lesions on development and growth of carcinogen-induced mammary tumors in the female rat. *Cancer Res.* **29,** 1541 (1969).
16. C. W. Welsch and J. A. Clemens, 6-methyl-8-β-ergoline-acetonitrile-induced inhibition of mammary hyperplastic alveolar nodular development and growth in C3H/HeJ female mice. *Proc. Soc. exp. Biol. (Med.)* **142,** 1067 (1973).

17. K. B. DeOme, L. J. Faulkin, H. A. Bern and P. B. Blair, Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.* **19,** 515 (1959).
18. J. Meites and C. S. Nicoll, Adenohypophysis: prolactin. *Ann. Rev. Physiol.* **28,** 57 (1966).
19. C. W. Welsch, H. Nagasawa and J. Meites, Increased incidence of spontaneous mammary tumors in female rats with induced hypothalamic lesions. *Cancer Res.* **30,** 2310 (1970).
20. J. E. Bruni and D. G. Montemurro, Effect of hypothalamic lesions on the genesis of spontaneous mammary gland tumors in the mouse. *Cancer Res.* **31,** 854 (1971).
21. C. W. Welsch and J. Meites, Effects of reserpine on development of 7, 12-dimethylbenzanthracene induced mammary tumors in female rats. *Experientia* **26,** 1133 (1970).
22. C. W. Welsch and C. Gribler, Prophylaxis of spontaneously developing mammary carcinoma in C3H/HeJ female mice by suppression of prolactin. *Cancer Res.* **33,** 2939 (1973).
23. J. A. Clemens and C. J. Shaar, Inhibition by ergocornine of initiation and growth of 7, 12-dimethylbenzanthracene-induced mammary tumors in rats: effect of tumor size. *Proc. Soc. Exp. Biol. (Med.)* **139,** 659 (1972).
24. R. Yanai and H. Nagasawa, Inhibition by ergocornine and 2-Br-α-ergocryptin of spontaneous mammary tumor appearance in mice. *Experientia (Basel)* **27,** 934 (1971).
25. O. Mioduszewska, T. Kaszarowski and C. Groski, The influence of hormones on breast cancer *in vitro* in relation to the clinical course of the disease. In *Prognostic Factors in Breast Cancer* (Edited by A. P. M. Forrest and P. B. Kunkler) p. 347, Williams & Wilkins, Baltimore, (1968).
26. H. Salih, H. Flax, W. Brander and J. R. Hobbs, Prolactin dependence in human breast cancers. *The Lancet* **ii,** 1103 (1972).
27. R. M. L. Murray, G. Mozaffarian and O. H. Pearson, Prolactin levels with L-dopa treatment in metastatic breast carcinoma. In *Prolactin and Carcinogenesis,* (Edited by A. R. Boyns and K. Griffiths) p. 158, Alpha Omega Alpha, Cardiff, Wales (1972).
28. R. P. Dickey and J. P. Minton, Levodopa relief of bone pain from breast Cancer. *New Eng. J. Med.* **286,** 843 (1972).
29. C. W. Welsch and M. J. Brennan, DNA synthesis of human breast carcinoma *in vitro:* influence of prolactin. (Manuscript in preparation).
30. B. A. Stoll, Hormonal management of advanced breast cancer. *Brit. med. J.* **2,** 293 (1969).

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Thymus-Histone Treated BHK21 Cells; Malignant Characteristics *in vitro* but Reduced Tumorigenicity

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Abstract—*BHK21/C13 cells have been treated in maintenance medium with bullock thymus histones. This has produced a line of cells, which we have designated BHK21/C13/HT, capable of continuous sub-culture, and which shows virtually all the* in vitro *characteristics associated with malignantly transformed cells. These include completely disordered growth pattern, growth in semi-solid agar, growth in the presence of low concentrations of serum, as well as high cloning efficiency. In spite of this, the cell line produced has been shown to be considerably less tumorigenic than the original BHK21/C13 cells. Its properties have been compared with BHK21/C13/Py6 cells. The evidence presented suggests that the appearance of the HT cells is due to transformation and not to selection.*

Possible mechanisms, by which exogenous histone could interact with cultured cells, as well as reasons which lead to the breakdown in the dissociation between in vitro *and* in vivo *malignancy are discussed.*

INTRODUCTION

IT HAS BEEN shown that rat liver histones acting on cultured cells in maintenance medium can produce the changes which occur in so-called "malignant transformation" [1], viz loss of cellular contact inhibition, multinucleated cells, giant cells and cells of bizarre shape. More recent studies have shown that BHK21 cells pretreated with rat liver histones exhibited markedly increased malignant characteristics, i.e. invasiveness and metastasis, when injected subcutaneously into a hamster. In fact, the untreated BHK21 cells neither invaded nor metastasized [2].

These observations prompted us to look at the effect of histones from other sources and it was noticed that after incubation of cells with bullock thymus histones in maintenance medium, abnormal colonies arose on returning the treated cells to growth medium.

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This paper describes the isolation of these variant cells, designated BHK21/C13/HT (subsequently referred to as HT cells) and the unusual properties they presented when compared to the original parent cells BHK21/C13, and also a viral transformed derivative BHK21/C13/Py6 (subsequently referred to as BHK21 and Py6 cells respectively). *In vitro*, the properties of the HT cells were typical of "malignantly transformed" cells [3], in that they grew in medium containing semi-solid agar, or low concentrations of serum, and plated out at a high efficiency without feeder layer. However, *in vivo*, they showed a much reduced tumorigenicity both in the time of appearance of the tumour and the type of growth produced.

The possible origins of these cells could be from either endogenous variants selected by the histones, or as transformants resulting from the specific experimental conditions employed. Although toxicity was apparent after histone treatment, the results obtained

with the growing cells in the resultant control and treated cultures, suggest that variants with the described properties were not present in the population prior to treatment.

MATERIAL AND METHODS

Tissue culture

The BHK21 cells used in our observations had been grown originally through 138 generations when received into the laboratory. Sublines of this preparation were set up, and stored in 15% dimethylsulphoxide (DMSO) in growth medium at −70°C. No BHK21/C13 subline was grown for more than 66 generations. The HT and Py6 were stored in a similar manner to the BHK21 cells. A generation was taken to mean the doubling of the entire cell population in log phase growth. Obviously, this parameter only serves as an approximate guide to the age of the cells because no measurements of the number of non-dividing cells in the population were made.

Using 2 oz glass medical flats, cells were grown to confluence in 10 ml growth medium containing 80% (v/v) Eagles Medium—Dulbecco's Modification (Flow Laboratories, Irvine, Scotland), 20% (v/v) calf serum (Flow Laboratories), 0·47 mg/ml glutamine, 2·95 mg/ml $NaHCO_3$, 500 units/ml penicillin G, 0·25 mg/ml streptomycin sulphate and 60 units/ml mycostatin. The whole was equilibrated with an atmosphere of 20% CO_2 in air and placed in an incubator at 37°C. Prior to harvesting cells, the confluent monolayer was washed with 10 ml phosphate buffered saline (PBS) [4]. Passage of the cells was carried out by harvesting with 5 ml 0·125% (w/v) trypsin (Flow Laboratories 1 :250) in PBS, and subcultures were set up at 1 :10 dilution. Each bottle produced approximately 10^7 cells. Cell concentrations were measured using an electronic cell counter (Celloscope, Grant Instruments, Cambridge, U.K.), or haemocytometer.

Growth medium containing 0·3% semi-solid agar was prepared by mixing equal volumes of 0·6% liquid agar and twice concentrated growth medium at 45°C. After cooling this mixture to 37°C, cells were introduced in a small volume, thus having little effect on the final agar concentration.

Photographs of cells were obtained from cultures grown in optically flat perspex petri dishes (Falcon, Biocult Laboratories, Paisley, Scotland). These cultures were fixed with 50% ethanol, stained with haematoxylin and eosin and mounted in glycerine jelly.

Histone preparation

Histones were prepared from bullock thymus using a technique described by Bonner and Marushige [5]. This method involved extraction of the basic nuclear proteins from chromatin with 0·25 *N* HCl and subsequent precipitation in 90% (v/v) aqueous acetone at 4°C. The resultant material was washed with acetone, vacuum dried and stored in a vacuum dessicator at 4°C.

All preparations were characterised by their ultraviolet absorption spectra and by the patterns obtained after electrophoresis on polyacrylamide gel [6]. The electrophoretic patterns gave four distinct bands which were closely similar to those reported for calf thymus histones [6]. Also, analysis of the preparations for DNA [7] proved negative, and amino acid analysis was closely similar to that published by Johns [8] for calf thymus histones.

Histone treatment of BHK 21 cells

A confluent monolayer of cells was washed three times with 10 ml PBS. Five millilitres of medium 199 (Burroughs Wellcome Type TC22, Beckenham, Kent) containing antibiotics and 2·2 mg/ml $NaHCO_3$ were then added. Bullock thymus histones were present in the treated cultures at concentrations of 25–100 μg/ml. The treated and untreated cultures were incubated at 37°C under an atmosphere of 5% CO_2 in air for 2 days. It should be noted at this stage that the above culture medium was different from that described in the previous section for the growth of cells. This type of maintenance medium was initiated in previous work [1] and was found to be the minimum essential medium necessary for survival of the cells as a *non-growing population*. The absence of serum also reduced the possibility of complex interactions between the negatively charged serum proteins and the positively charged histones.

Determination of plating efficiency and percentage transformation

Dilutions of trypsinized cell suspensions were made in growth medium so that a known number of cells (500–1000) could be seeded into 5 cm plastic gridded petri dishes. The dishes were incubated for 8–10 days in a sealed dessicator containing an atmosphere of 20% CO_2 in air. After this time, the cultures were fixed, stained, and mounted as described previously. The cultures were examined with a stereoscopic microscope, and the numbers of normal and variant colonies noted. The ratio of the total number of colonies formed to the

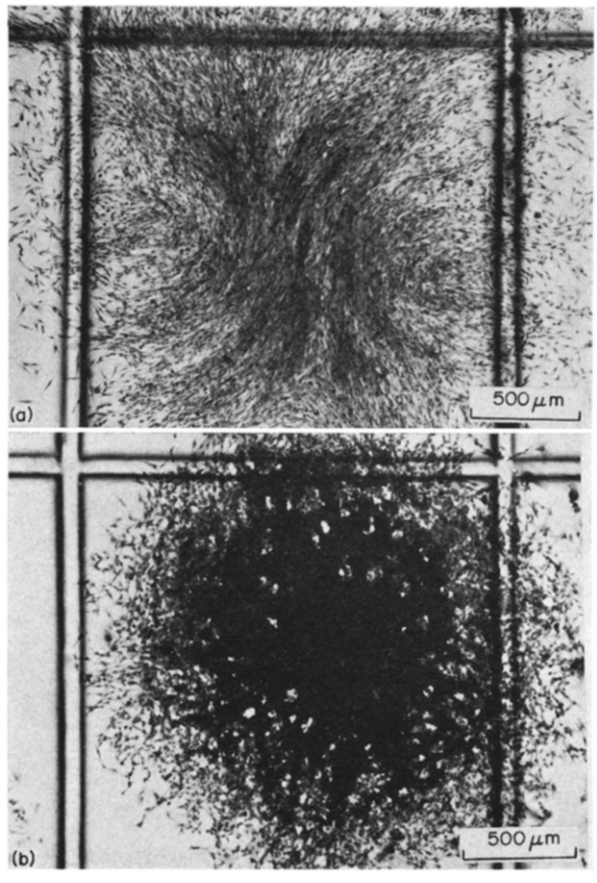


Fig. 1. *Colonies obtained after the incubation of BHK*21 *cells for* 2 *days in tissue culture medium* 199 *containing* 100 *μg/ml bullock thymus histone. After treatment, cells harvested with trypsin as previously described and* 500–1000 *cells subcultured for* 8–10 *days in growth medium containing no histone.* (a) *colony of normal BHK*21 *type,* (b) *colony of transformed type.*

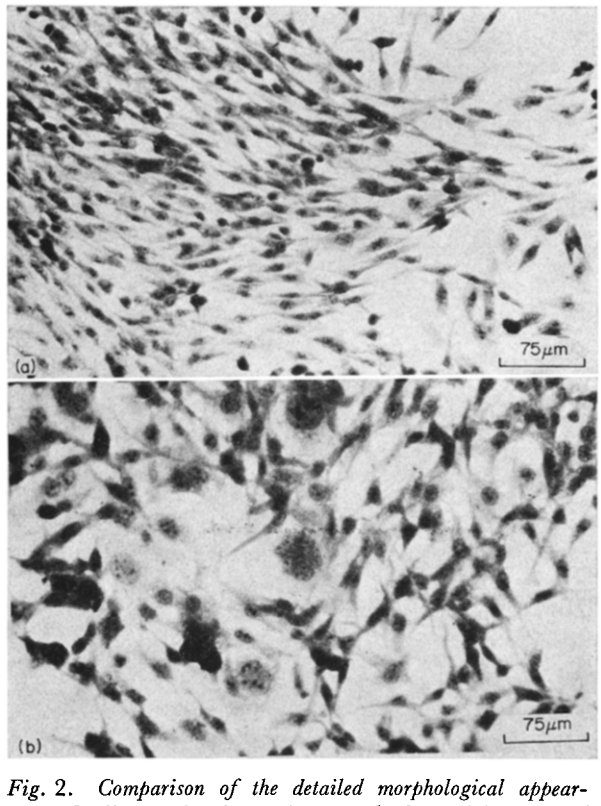


Fig. 2. *Comparison of the detailed morphological appearance of cells growing in continuous subculture.* (a) *untreated BHK*21 *cells,* (b) *transformed HT cells.*

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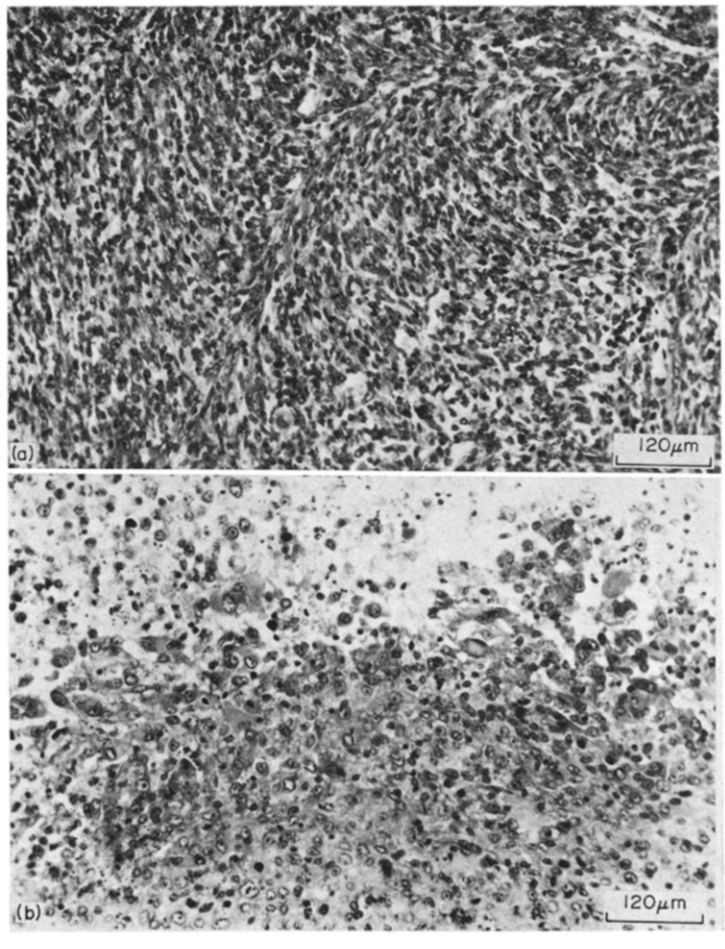


Fig. 5. *Histological appearance of typical primary tumours resulting from the subcutaneous inoculation of* (a) *BHK*21 *cells*, (b) *HT cells. H and E.*

total number of cells in the original inoculation in the petri dish expressed as a percentage, was termed plating efficiency.

The expression percentage transformation was defined as the percentage of the total colonies observed exhibiting breakdown in parallel alignment and dense heaped-up populations of cells [9].

Estimation of growth rate in 2% calf serum

Replicate cultures were prepared with a number of 2 oz glass medical flats seeded with $0{\cdot}6\times10^6$ cells in 10 ml of growth medium containing 2% calf serum; the gas phase of each bottle was flushed out with 20% CO_2 in air. At 24 hr intervals, the total cell populations from 2 bottles were harvested and counted individually with the cell counter. In some experiments, the medium and gas phase in each bottle were replaced each day.

Implantation of cells into the hamster

Syrian hamsters, aged 3–6 months (Wrights of Essex) were lightly anaesthetised with ether and 1 ml cell suspensions, containing 2×10^5 cells (BHK21, Py6 or HT), were injected subcutaneously into each animal's left dorsolumbar region. Great care was taken during this procedure to avoid puncturing the body wall.

The times at which the tumours were first detected were noted and some animals sacrificed between 4 and 13 weeks after inoculation if the tumours were thought to be causing distress. The remaining animals injected with BHK21 or HT cells were sacrificed 13 weeks after the inoculation date, whereas those animals injected with Py6 cells were sacrificed after 7 weeks. The primary tumours were dissected out, the weight of each determined, and the animals explored for invasion and metastases. Samples of the primary tumours were taken for histological examination.

RESULTS

The transformation of BHK21 cells by bullock thymus histones

(a) *The origins of BHK21/C13/HT cells.* A change in the growth characteristics of some of the hamster kidney cells became apparent after exposure to bullock thymus histones in maintenance medium and the return of the cells to growth medium. In the latter, the cell morphology and growth pattern in individual colonies now fell into one of two types. One consisted of spindle-shaped cells arranged in parallel lines and whorls (Fig. 1a), which was identical to that produced by untreated BHK21 cells; and the other showed transformed morphology (Fig. 1b) very reminiscent of that obtained after treatment of BHK21 cells with polyoma virus [9]. The cells grew in completely disordered criss-cross fashion to form a dense three-dimensional network. Individual cells grew across each other with apparent lack of contact inhibition of movement. There was little tendency for cells to move from the edges of the colonies, or for the cells to pack in any ordered array. The colonies were easily recognisable, even by eye, because of the increased density of staining which resulted from the piled up heaps of cells. Transformation of the type described was obtained in treated cultures from 10 out of 16 experiments. The untreated cultures contained no colonies of variant cells.

(b) *The morphology of BHK21/C13/HT cells in tissue culture.* All the successfully transformed cultures exhibited similar properties in continuous subculture. Figure 2(a) and 2(b) show typical examples of cultures of untreated BHK21 cells and HT cells respectively at high magnification. It can be seen in Fig. 2(b) that there is a complete breakdown in contact inhibition and extensive multilayering. Giant cells are present which are either multinucleate or which contain one very large nucleus. These characteristics are not seen in the BHK21 culture (see Fig. 2a). Only with increasing cell density did the HT cells tend to change to an epithelioid shape with small amounts of poorly defined cytoplasm, and, at confluence, this tendency to round up became extreme and the whole surface was covered by cells of this type.

(c) *Dose–response relationship for BHK21 cells treated with histones.* All data given in this section were obtained from suspensions of trypsin harvested BHK21 cells obtained immediately after histone treatment. Figure 3(a) gives the number of cells recovered after treatment of BHK21 cells with histones. The cell number prior to incubation with medium 199 with or without histones was on average $10{\cdot}7\times10^6$ (S.D. $\pm1{\cdot}9\times10^6$; 10 observations). Incubation in 199 alone reduced this figure to $7{\cdot}7\pm2{\cdot}6\times10^6$ cells (10 observations). The presence of 25 μg/ml of histones only slightly reduced this to $6{\cdot}9\pm1{\cdot}9\times10^6$ cells (3 observations) but the higher concentrations of 50 μg/ml and 100 μg/ml resulted in considerable cytolytic toxicity and only $2{\cdot}6\pm0{\cdot}8\times10^6$ cells (7 observations) and $2{\cdot}2\pm1{\cdot}4\times10^6$ cells (10 observations) respectively remained after these levels of treatments.

The plating efficiencies of 500–1000 histone treated BHK21 cells, after 8–10 days incubation

in a petri dish containing growth medium and no histone, are illustrated in Fig. 3(b). After pretreatment with 199 alone, the BHK21 cells gave a plating efficiency of 4·8±1·4% (9 observations), which is lower than that obtained for cells harvested from growth medium, 12·4±5·5% (13 observations), and presumably resulted from the lack of serum in the pretreatment medium. Treatment with 199 containing 25 μg/ml of histones lowered the plating efficiency of the BHK21 cells even further to

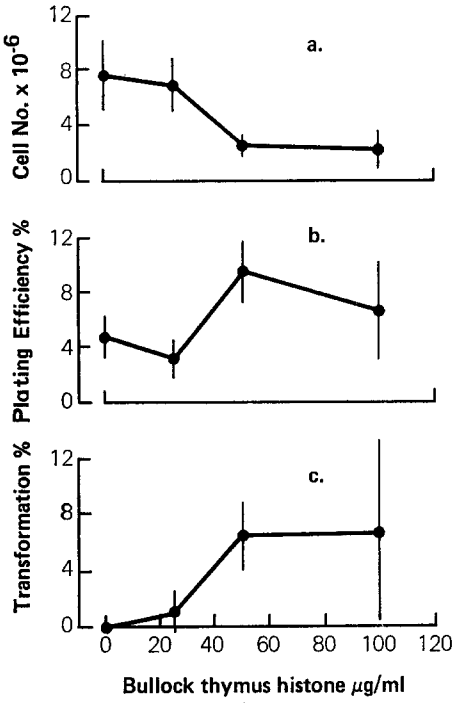


Fig. 3. Effect of incubation of BHK21 cells with different concentrations of bullock thymus histone on the (a) *cell number,* (b) *plating efficiency,* (c) *percentage transformation Cell numbers were measured on trypsinized suspensions of cells immediately after removal of the histone. Plating efficiency and percentage transformation were measured on colonies which were obtained in a manner identical to that described in Fig. 1. Each value is given as a mean ±S.D.*

3·2±1·3% (4 observations) but treatment with the higher concentrations of histones, 50 and 100 μg/ml, raised the plating efficiencies to 9·4±2·2% (4 observations) and 6·7±3·5% (4 observations) respectively.

Figure 3(c) demonstrates the relationship between histone dose and percentage transformation in the same cultures as those described in Fig. 3(b). No variant colonies were identified in the untreated cultures. After histone treatment of 25 and 50 μg/ml, the percentage transformation was 1·1±1·3% (4 observations) and 6·4±2·4% (4 observations) respectively. Exposure to 100 μg/ml histone only resulted in a slightly higher percentage transformation of 6·9±6·5% (4 observations).

The properties of BHK21 cells and its derivatives in vitro

Variant cells from previously histone treated cultures vigorously outgrew normal cells and after 66 generations in growth medium still maintained the same morphology and growth pattern as illustrated in Figs. 1(b) and 2(b). Unless otherwise stated, all results in this section with the HT cells were obtained on populations which had been subcultured through at least 40 generations.

(a) *Growth in medium containing 2% calf serum.* BHK21 cells showed no growth in medium containing 2% calf serum (Fig. 4). As expected [10, 11], the polyoma transformed cells grew

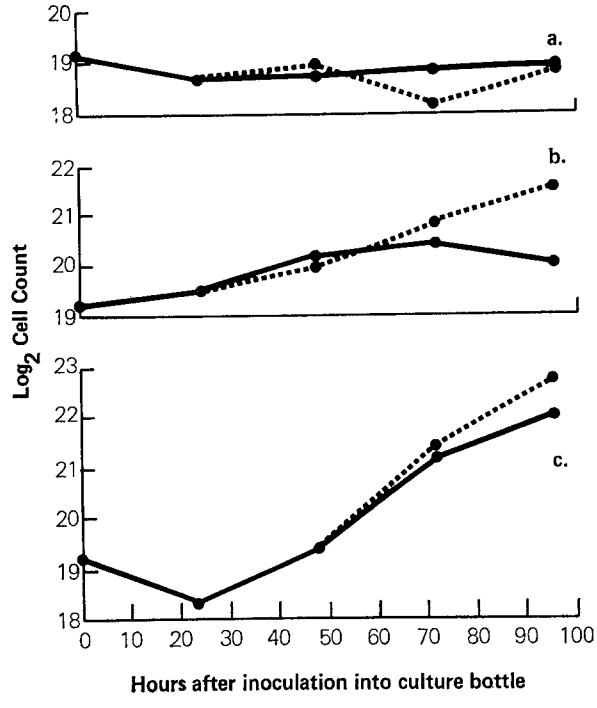


Fig. 4. The growth of (a) *BHK21 cells,* (b) *HT cells, and* (c) *Py6 cells in medium containing 2% calf serum. The measurements on the HT cells were carried out on a population of cells which had been grown for 40 generations in culture, and contained no detectable BHK21 cells. Broken line is growth curve from cultures in which medium and gas changed each day after 24 hr in culture.*

very well in the presence of this low concentration of serum. The HT cells grew less well than the Py6 cells in this medium, but still doubled their number within 48 hr, and, if the medium were changed every day, grew vigorously.

(b) *Growth in semi-solid agar.* The BHK21 cells did not grow in medium containing 0·3% semi-solid agar, whether pre-incubated with medium 199 or transferred directly from growth medium, whereas an inoculum of 10^6 HT cells grew very well in the agar/medium environment and increased their number by fourfold after 3 days incubation (Table 1).

After histone treatment of the BHK21 cells, the proportion of variant to normal cells in the final growing population varied considerably. Thus, 0·3% agar/medium was used as a rapid technique for the isolation of the variant cells. After treating mixed BHK21/HT cell cultures in this way, more than 95% of the colonies showed transformed morphology on plating out.

Like HT cells, Py6 cells grew in medium containing semi-solid agar. This observation has been reported previously by others [12].

Table 1. *Growth of BHK*21 *and HT cells in growth medium containing semi-solid agar*

Cell type	Initial inoculum ($\times 10^{-6}$)	*Final cell number ($\times 10^{-6}$)
BHK21	1·1	1·2
HT	1·0	4·1

*Cell numbers estimated in cultures after 3 days incubation at 37°C in growth medium containing 0·3% agar. Each value given is the average from three separate experiments.

(c) *Growth in mixed culture.* Mixtures containing 95% BHK21 cells and 5% HT cells (designated 95/5), and 50% BHK21 cells and 50% HT cells (designated 50/50) were made in growth medium, and grown in continuous culture. After a known number of generations, aliquots of the mixture cultures were diluted, plated out and assayed for colonies of normal and transformed morphology.

Neither of the mixed cultures showed large increase in number of variant colonies until after 33 generations, when they accounted for 85% of the colonies in the 50/50 culture and 52·5% in the 95/5 culture. No normal colonies were seen in the 50/50 culture after 53 generations, and only 3% in the 95/5 after 63 generations (Table 2).

(d) *Plating efficiencies.* The plating efficiency of BHK21 cells harvested from growth medium was 12·4 ±5·5% (13 observations), whereas the plating efficiency of HT cells measured under similar conditions was 22·5 ±5·3% (7 observations). This was very similar to the plating efficiency of Py6 cells which was found to be 22·0 ±2·6% (9 observations).

Table 2. *Growth of HT cells in mixed culture*

*Number of generations	‡Percentage †50/50	Transformation †95/5
16	52·5	7·0
33	85·0	52·5
53	100·0	73·9
63	—	97·0

*Number of generations estimated from time original mixture prepared.

†Mixtures containing 50% BHK21 cells and 50% HT cells, and 95% BHK21 cells and 5% HT cells designated 50/50 and 95/5 respectively.

‡Percentage transformation represents the percentage of the total colonies exhibiting a transformed growth pattern.

*The growth of BHK*21 *cells and its derivatives* in vivo

Table 3 gives the tumour incidences, mean tumour wet weights, mean growth periods, and mean ratios of tumour weight to growth period after the subcutaneous injection of BHK21, Py6 and HT cells into hamsters. The tumour incidences in the animals implanted with HT cells were much lower after 5 and 6 weeks than in those implanted with BHK21 and Py6 cells. After 13 weeks, there was no significant difference between tumour incidences for all groups. The mean ratio of tumour weight to growth period of the tumours from the HT cells was significantly lower than those of the tumours from the Py6 cells ($0·01 > P >$

Table 3. *Tumour incidence in Syrian hamsters after subcutaneous injection of* 2×10^5 *BHK*21, *Py*6, *or HT cells*

Cell type	Tumour incidence (weeks after injection)				Mean weight of tumour (g ± S.D.)	Mean growth period of tumour (weeks ± S.D.)	Weight of tumour / growth period (Mean ± S.D.)
	4	5	6	13			
BHK21	0/18	8/18	9/18	14/18	8·2 ± 4·2	9·1 ± 1·6	1·0 ± 0·5
Py6	4/5	4/5	5/5	5/5	7·0 ± 2·2	5·8 ± 1·6	1·2 ± 0·1
HT	0/16	1/16	2/16	13/16	6·5 ± 4·6	11·7 ± 1·5	0·6 ± 0·4

The HT cells used had been grown for 46 generations since their isolation.

0·002), and the BHK21 cells ($0·05 > P > 0·02$). Invasive tumours and secondaries were only found in those animals containing a primary tumour grown from Py6 cells.

The BHK21 cells produced well defined encapsulated tumours which were consistently solid. In cross section they contained cells which appeared in whorls reminiscent of the appearance of similar cells in monolayer culture. An example of the histological appearance of these tumours is given in Fig. 5(a). The nuclei were consistent in size, shape and staining density. There were occasional haemorrhagic areas and in these locations there were necrotic tumour cells and a few mononuclears.

Tumours derived from the HT cells were very soft and haemorrhagic and in cross-section there was little evidence of organisation, the cells apparently growing in a haphazard manner (see Fig. 5b). The nuclei were variable in size, shape and staining density. In young or small tumours, there was always at least 50% of the areas necrotic. In larger or older tumours, the central necrosis and haemorrhage extended almost to the periphery of the tumours so that the tumours appeared as a blood sac. In all stages of development, numerous cells of the reticuloendothelial system (RES) of all types were visible, and histologically at least, the tumours appeared to be in a state of rejection and lysis.

The Py6 cells also produced solid tumours containing cells which again in cross-section appeared to be orientated in whorls but there was considerable variation in the size, shape and staining intensity of their nuclei. Compared with parent BHK21 cells, there appeared to be much more fibrous ground substance between cells. Frequently, there was central necrosis, but no haemorrhage, and it was in these areas where the fibrous nature of the tumours was most pronounced. There was little evidence of RES cells.

DISCUSSION

In much of the literature, there has been an unfortunate confusion between tumorigenicity and malignancy. The latter can only be recognised by virtue of the fact that a tumour *in vivo* invades and/or metastasizes. In the clinical situation, there may be presumptive evidence that a given primary tumour would be capable of invading and metastasizing but has not yet done so. So-called "malignant transformation" *in vitro* has often been described in cells which, when injected into animals, are definitely tumorigenic but show no real evidence of malignancy. In fact, such tumours are frequently encapsulated, as was the case with our untreated BHK21 cells. Although we have, for convenience, already used the term "malignant transformation" to indicate a specific type of behaviour of cells *in vitro*, it must be made very clear that the subsequent discussion deals with the phenomenon of tumorigenicity and not necessarily with malignancy itself.

In the present studies, we have induced the appearance of a variant cell (HT) from an established cell line, BHK21/C13, by treatment of an non-dividing culture with bullock thymus histone. It is well known that viruses [9, 13] and mycoplasma [14] transform BHK21 cells in culture, but investigations on both the histone solution and the transformed cells did not result in the detection of foreign bacteria or viruses. The latter, of course, can never be completely excluded.

The mechanisms by which exogenous histone can influence the biochemistry of cultured cells still remain obscure. Some evidence exists for the uptake of histone by the cytoplasm of the cell [15–17], but whether this material reaches the nucleus is not definitely known, and the possibility that it is incorporated into the recipient nucleohistone is to some extent still pure speculation. However, some recent work has implied that the exogenous histone at least affects nuclear size [18]. In mouse kidney organ culture, exogenous histones can modify the lactate dehydrogenase isoenzyme pattern [19]. This modification is not only specific for the type of histone used, but also tends towards the isoenzyme pattern of the organ from which the histone was derived. One very likely interpretation of these results could be alteration of gene expression by the added histone, and this would suggest uptake into the nucleus. This idea receives further support from the findings that calf thymus histones and histone subfractions, when added to sub-confluent mouse fibroblast cells, cause significantly higher cell densities at confluence than in the control culture, and disruption of the normal ordered arrangement of cells. These changes were seen only on application of histones to growing cells, but not confluent cells. It was, therefore, suggested that it is possible that exogenously added histone interacted with replicating DNA [20].

It is, of course, also possible that the observed cell transformation was not the result of histones entering the cells and directly producing an effect. Histones might affect other molecules and their behaviour in the cell. Many studies have shown that histones

enhance the cellular uptake of macromolecules such as RNA [21], viral RNA [22], and proteins such as albumin [15, 23].

Of all the cellular properties which can be transformed *in vitro* by viruses, chemicals, or radiation, most emphasis has been placed on the relationship between changes in growth characteristics of cell population and their tumorigenic behaviour. This has included not only the process by which cells increase their numbers but also their morphological interactions while carrying out this process. Many researchers have successfully correlated increased tumorigenicity with the appearance of random growth characteristics *in vitro* [24–29], however, the behaviour of the HT cells did not support this concept. In culture, they showed all the properties typical of random growth, including the ability to grow in conditions which would normally be inhibitory [11, 13], but in the animal, their tumorigenicity was very low. Dissociation between *in vitro* growth control and tumorigenicity has been previously reported. Only in one publication [30] was the dissociation of the HT cell type, whereas in the vast majority of cases [31–35], the dissociation observed was the converse of that found for HT cells, i.e. a high degree of *in vitro* growth control and also high tumorigenicity. Defendi *et al.* [31] found that some sublines from BHK21/C13 have many morphological characteristics of other "normal" diploid cells, but had tumorigenic ability at very low inoculum. Similarly, Di Paolo *et al.* [35] found that hamster embryonic cells grown in 3-methyl-cholanthrene underwent neoplastic transformation. When injected into homologous hosts they produced tumours, but *in vitro* exhibited little morphological change. The reasons for this type of dissociation between *in vitro* and *in vivo* properties of cells may lie with the techniques used for their study, and the inherent inability to control all the parameters. In the tissue culture of cells, variants are constantly being thrown off into the general population [33] and this could easily explain the many reports of high tumorigenicity in lines which have high growth control *in vitro*.

It is more difficult to explain lowered tumorigenicity of cells in culture, showing so-called "malignant transformation", as was the case with the HT cells. Possible explanations are provided if one admits lack of control over all the parameters and in some cases pure ignorance of their possible existence. Two such parameters are antigenicity and *in vivo* growth rate. Aaronson and Todaro [30] concluded that the reduced tumorigenicity of Balb/3T12 cells after "malignant transformation" by polyoma virus was most likely due to increased antigenicity, because they found that the inoculated animals developed small nodules in the first two weeks, most of which then regressed. No such growth pattern was observed in the *in vivo* development of HT cell tumours. However, in the cell-containing blood sac, which eventually emerged from the inoculated HT cells, there were numerous RES cells and this alone suggested that the transformed cells elicited an immunological response from the host. Since an immunological response was by no means obvious in the control tumours from BHK21 cells, it seems likely that the histone treatment had produced a new antigen which could survive subculture. In other words, the histone must have affected genetic mechanisms, which suggests direct action on the nucleus itself.

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REFERENCES

1. A. L. Latner and E. Longstaff, Transformation of mammalian cells by crude histones. *Brit. J. Cancer* **25,** 280 (1971).
2. A. L. Latner, E. Longstaff and G. A. Turner, Enhanced malignancy of cells transformed by rat liver histone. *Brit. J. Cancer* **27,** 218 (1973).
3. I. Macpherson, The characteristics of animal cells transformed *in vitro*. *Advanc. Cancer Res.* **13,** 169 (1970).
4. R. Dulbecco and M. Vogt, Plague formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99,** 167 (1954).
5. K. Marushige and J. Bonner, Template properties of liver chromatin. *J. Roy. micr. Soc.* **80,** 47 (1966).
6. E. W. Johns, The electrophoresis of histones in polyacrylamide gel and their quantitative determination. *Biochem. J.* **104,** 78 (1967).

7. 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).
8. E. W. Johns, The preparation and characterisation of histones. In *Histones and Nucleohistones*, (Edited by D. M. P. Phillips) p. 12, Plenum Press, New York (1971).
9. M. Stoker and I. MacPherson, Studies on transformation of hamster cells by polyoma virus *in vitro*. *Virology* **14,** 359 (1961).
10. R. R. Burk, Growth inhibitor of hamster fibroblast cells. *Nature (Lond.)* **212,** 1261 (1966).
11. C. P. Stanners, J. E. Till and L. Siminovitch, Studies on the transformation of hamster embryo cells in culture by polyoma virus. I. Properties of transformed and normal cells. *Virology* **21,** 448 (1963).
12. I. MacPherson and L. Montagnier, Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology* **23,** 291 (1964).
13. W. A. Strohl, A. S. Rabson, and H. Rouse, Adeno-virus tumorigenesis: role of the viral genome in determining tumour morphology. *Science* **156,** 1631 (1967).
14. I. MacPherson and W. Russell, Transformations in hamster cells mediated by mycoplasmas. *Nature (Lond.)* **210,** 1343 (1966).
15. H. J. P. Ryser and R. Hancock, Histones and basic polyamino acids stimulate the uptake of albumin by tumour cells in culture. *Science* **150,** 501 (1965).
16. F. F. Becker and H. Green, The effects of protamines and histones on the nucleic acids of ascites tumour cells. *Exp. Cell Res.* **19,** 361 (1960).
17. A. S. Levine, M. E. Nesbit, J. G. White and J. W. Yarbro, Effects of fractionated histones on nucleic acid synthesis in 6C3HED mouse ascites tumour cells and in normal spleen cells. *Cancer Res.* **28,** 831 (1968).
18. A. L. Latner, E. Longstaff and J. M. Lunn, Influence of histones on nuclear size. *Nature (Lond.)* **243,** 107 (1973).
19. A. L. Latner and E. Longstaff, Modification by crude histones of gene activity for lactate dehydrogenase. *Nature (Lond.)* **224,** 71 (1969).
20. G. M. Kolodny, Histone mediated changes in morphology and confluent density of cultured cells. *J. cell. Physiol.* **81,** 233 (1973).
21. H. Amos and K. E. Kearns, Influence of bacterial ribonucleic acid on animal cells in culture. II. Protamine enhancement of RNA uptake. *Exp. Cell. Res.* **32,** 14 (1963).
22. C. E. Snull and E. H. Ludwig, Enhancement of Plaque-forming capacity of polio virus ribonucleic acid with basic proteins. *J. Bact.* **84,** 1035 (1962).
23. H. J. P. Ryser, Uptake of protein by mammalian cells: an underdeveloped area. *Science* **159,** 390 (1968).
24. M. Vogt and R. Dulbecco, Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. nat. Acad. Sci. U.S.* **49,** 171 (1963).
25. Y. Berwald and L. Sachs, *In vitro* transformation of normal cells to tumour cells by carcinogenic hydrocarbons. *J. nat. Cancer Inst.* **35,** 641 (1963).
26. J. A. Dipaolo, J. A. Nelson and P. J. Donovan, Morphological, oncogenic and karyological characteristics of Syrian hamster embryo cells transformed *in vitro* by carcinogenic polycyclic hydrocarbons. *Cancer Res.* **31,** 1118 (1971).
27. F. K. Sanders and B. O. Burford, Morphological conversion of cells *in vitro* by *N*-nitrosomethylurea. *Nature (Lond.)* **213,** 1171 (1967).
28. S. Mondal and C. Heidelberger, *In vitro* malignant transformation by methylcholanthene of the progeny of single cells derived from C34 mouse prostate. *Proc. nat. Acad. Sci. U.S.* **65,** 219 (1970).
29. F. Rapp and R. Duff, *In vitro* transformation by herpes viruses. *Fed. Proc.* **31,** 1660 (1972).
30. S. A. Aaronson and G. J. Todaro, Basis for the acquisition of the malignant potential by mouse cells cultivated *in vitro*. *Science* **162,** 1024 (1968).
31. V. Defendi, J. Lehnan and P. Kraemer, Morphologically normal hamster cells with malignant properties. *Virology* **19,** 592 (1963).
32. T. Gotlieb-Stematsky and R. Shilo, Studies on the tumorigenic properties of baby hamster kidney cell lines and a method of selection of high and low tumorigenic clones. *Virology* **22,** 314 (1964).
33. O. Jarrett and I. MacPherson, The basis of the tumorigenicity of BHK21 cells. *Int. J. Cancer* **3,** 654 (1968).

34. J. WYKE, Phenotypic variation and its control in polyoma transformed BHK21 cells. *Exp. Cell Res.* **66,** 209 (1971).
35. J. DIPAOLO, A. RABSON, R. MALMGREN, *In vitro* viral transformation of chemical transformed cells. *J. nat. Cancer Inst.* **40,** 757 (1968).

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A New Purine Analogue with Antimitotic Activity

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Abstract—*The compound 4-benzylamino 6-methyl 7-benzyl pyrrolo [2, 3-d] pyrimidine has been found to arrest cultured cells in metaphase. The block can be released upon removal of the drug. However, after prolonged incubation with the inhibitor, some irreversible effects occur, such as anarchic reconstruction of the nuclear membrane around dispersed chromosomes and formation of multinucleated giant cells. In addition, the drug has some effect on the shape of interphase or resting fibroblasts and on mitochondria. Most of these effects can be explained as action at the level of microtubule organization.*

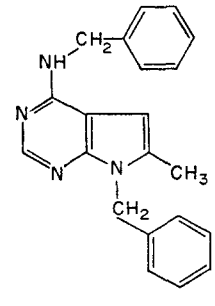
INTRODUCTION

SINCE several deaza-analogues of purines, like tubercidin [1], are endowed with antimitotic and antitumor action, we have undertaken synthesis of new purine analogues, pyrrolo-pyrimidines di-sustituted in positions 4 and 7, and looked for their biological activity. Our interest in such compounds was also reinforced by the finding that adenine derivatives are required for the full expression of transformed characters of BHK21 cells transformed by oncogenic viruses [2]. Therefore, we sought purine derivatives selectively suppressing transformed characters without impairing growth of normal cells.

In the course of these investigations, we found incidentaly that the compound, (code name BM 203) the structure of which is given opposite, had a strong inhibitory effect on growth of both normal and transformed cultured cells. Details of the procedure of synthesis of the compound and of the series to which it belongs, have been described elsewhere [3].

4 – Benzylamino 6 – methyl 7 – benzyl pyrollo [2,3–*d*] pyrimidine =

— BM 203 —



MATERIAL AND METHODS

The compound, which is water insoluble, was dissolved in dimethylsulphoxyde at a concentration of 10 mM. It was then diluted in saline at appropriate concentrations. Dilutions, exhibiting a fine precipitate, were immediately added to cultures of BHK21 hamster cells in ETC10 medium [4]. Several clones of BHK21 cells, transformed by Rous sarcoma virus, polyoma virus or a hamster pseudotype of mouse sarcoma virus, [5] were used as well as mouse and human embryo fibroblasts.

Cytological observations were carried out either on living cells by phase contrast microscopy or on fixed cells after staining with Giemsa solution.

Electron microscopy: cells in pellets were fixed by glutaraldehyde–osmium double fixation method and embedded in Araldite. Observations were made after stainin with lead citrate.

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RESULTS

In all cell systems used, a complete inhibition of growth was observed at the concentration of 5 μM (Fig. 1). At lower concentrations (1 and 2 μM), there was an initial, but transient inhibition.

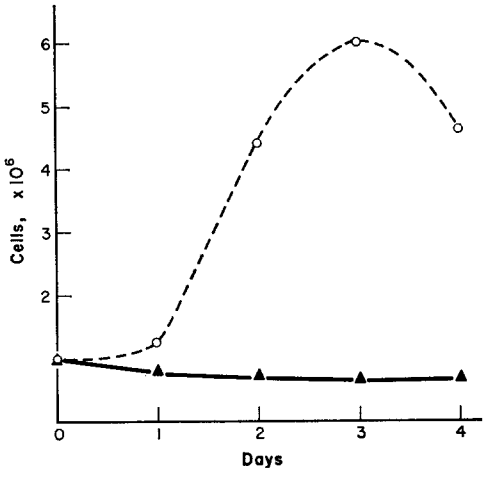


Fig. 1. Growth curve of BHK21 cells (10^6 cells seeded in 60 mn petri dishes counted after trypsination every 24 hr. ○—○ Control untreated cells, ▲—▲ With 5 μM BM 203.

Short term exposure to the drug (at 5 μM, less than 24 hr results in three kinds of effects, all of which are reversible:

(1) On interphasic or resting cells, cells which originally were elongated (fibroblasts), lose their shapes in a few hours after the addition of the compound and become round or polygonal. Progression of cells to mitosis is not impaired.

(2) In cells undergoing mitosis, blocking occurs at metaphase, (Fig. 2 and 3) and following a few hours of treatment, accumulation of metaphasic cells can be observed.

In these cells, mitotic spindles are no longer seen. At the ultrastructural level, a disorganization of spindle microtubules can be seen clearly, especially in the vicinity of the centrioles (Fig. 3a). The latter are frequently accompanied by atypically duplicated pericentriolar bodies.

As a consequence, mitotic cells remain round and refractile and can be detached easily from a cell monolayer.

(3) Alterations of mitochondria. These organelles are swollen and exhibit structural deficiencies: heterogeneous matrix, myelin figures, superabundant and irregularly arrayed cristae (Fig. 3c). Their sizes are very variable, some being enlarged and some being smaller with a dense matrix.

Accumulation of such dense degenerated mitochondria was frequently observed in some parts of the cytoplasm, resulting in autophagic vacuoles (Fig. 3b).

These three effects are fully reversible upon removal of the drug; interphasic fibroblasts regain their elongated shape in a few hours. In mitotic cells, the nuclear membrane is observable 4 hr after drug removal. Some cells are binucleated, indicating that mitosis may have ended without cytokinesis.

By 24 hr, examination in the electron microscope shows normal mitochondria with well-developed cristae. In the cytoplasm, intense synthethic activity is revealed by the abundant ergastoplasmic lamellae and a large number of vesicles surrounding dictyosomes.

Further evidence that cell viability is not affected by such a treatment, comes from measurement of their cloning ability in agarose gel [6, 7]. An exponentially growing culture of BHK21 cells transformed by hamster sarcoma virus was treated for 4 hr with 5 μM BM 203; mitotic cells were selectively detached by gently shaking the flask, then washed and plated in agarose at a concentration of $2 \cdot 10^4$ cells per 60 mm petri dish. The number of colonies obtained after 8 days was as high as that of the control cells (untreated, detached with trypsin).

Long-term effects: If the cells are left in the presence of the drug 24 hr or more, some irreversible changes appear: formation of multinucleated cells and nuclear pulverisation, probably caused by formation of nuclear membrane around individual chromosomes (Fig. 4). At this stage, cells could no longer divide, although they remained alive for up to one week.

DISCUSSION

It is known [8] that purines or purine derivatives at high concentration can block cells in metaphase, mimicking the effect of spindle poisons. A pyrimidine derivative was also reported to arrest cells in metaphase [9]. However, the high activity of the compound described here is remarkable and suggests a more specific mechanism of action. Apart from the effect on mitochondria, all the observed changes could be explained by some effect of the drug on microtubule organization. The effect on mitochondria and its complete reversibility remain to be explained. It is worth mentioning that we have observed a similar effect on mitochondria after treatment

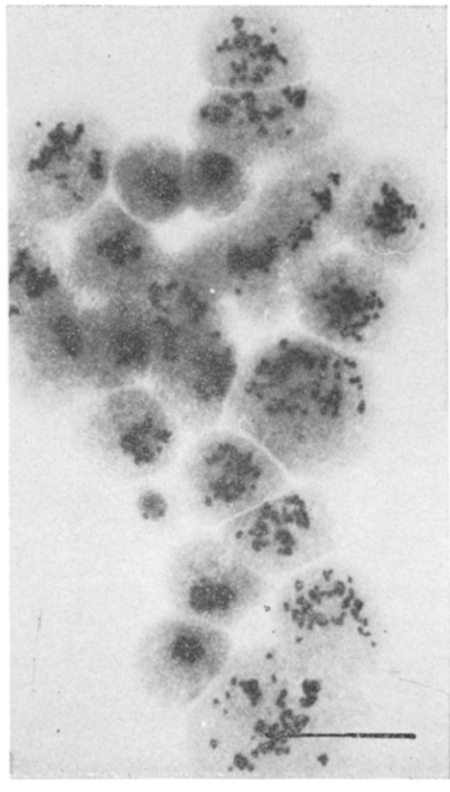


Fig. 2. *Mitotic BHK*21/13 *cells, obtained by gentle shaking of a monolayer treated with* 5 μM *BM* 203 *for* 4 *hr. Scale:* 50 μm *(Giemsa staining).*

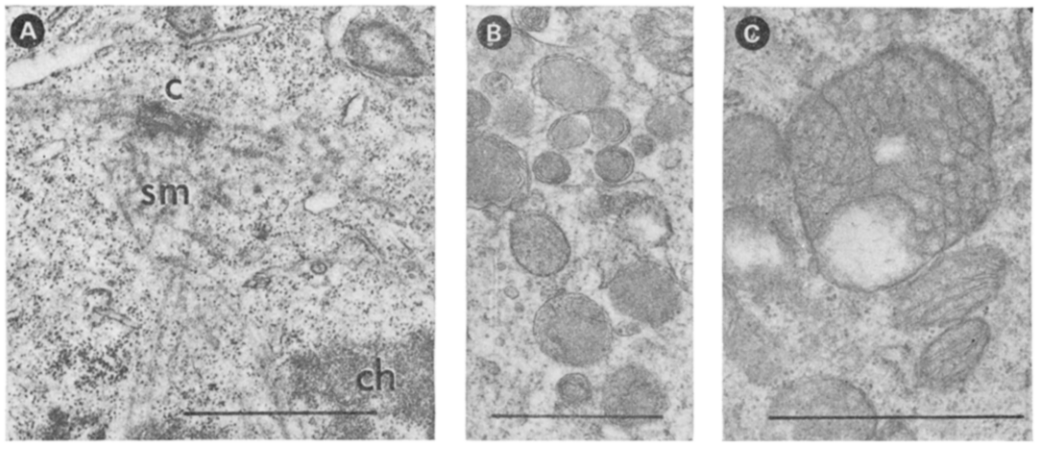


Fig. 3. *Electron micrographs of thin sections of BHK*21/13 *cells incubated with BM* 203 *for* 4 *hr.* A, *Portion of a cell showing a disorganization of spindle microtubules (sm) in the vicinity of the centriole (c).* B, *High magnification of an area with dense degenerated mitochondria.* C, *Section of mitochondria with superabundant and irregularly arrayed cristae. Ch = chromosomes. Scale:* 1 μm *in all figures.*

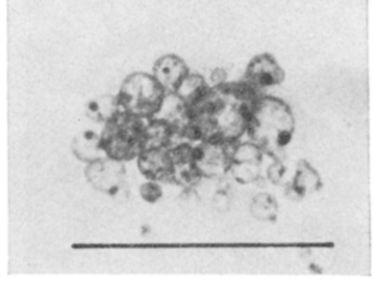


Fig. 4. *Multinucleated cell formed after a long-term incubation in the presence of BM* 203. *Scale:* 50 μm.

(to face p. 612)

of cells with vinblastine (Vinbé, Eli Lilly Laboratories, France) at a concentration of 0·1 μg/ml for 4 hr.

Studies on the relation between chemical structure and activity [3] had indicated that the two benzyle radicals, in their indicated positions, are essential for activity.

The drug may prove to be useful in *in vitro* studies to isolate mitotic cells, under reversible conditions of metaphase blocking.

Preliminary studies have shown that the drug was not toxic for mice, after intraperitoneal administration at a dose of up to 160 mg/kg.

REFERENCES

1. R. L. Tolman, R. K. Robin and L. B. Townsend. Pyrrolopyrimidine nucleosides II. The total synthesis of 7-β-B-ribofuranosyl pyrrolo [2, 3-d] pyrimidines related to Toyokamycine. *J. Het. Chemistry* **4,** 230 (1967).
2. L. Montagnier. Factors controlling the multiplication of unstransformed BHK21 cells under various environmental conditions. In *Growth Control on Cell Cultures*, (Edited by G. E. W. Wolstenholme and J. Knight) p. 33. Churchill-Livingstone, Edinburgh (1971).
3. J. P. Marquet, L. Montagnier, J. Gruest, J. D. Bourzat, J. André-Louisfert and E. Bisagni. Sur une nouvelle série d'analogues puriques à action antimitotique: relations structure–activité. *Bull. Chimie thérapeut.* **6,** 427 (1971).
4. I. Macpherson and M. Stoker, Polyoma transformation of hamster cell clones—An investigation of genetic factors affecting cell competence. *Virology* **16,** 147 (1962).
5. J. Zavada and I. Macpherson, Transformation of hamster cell lines *in vitro* by a hamster sarcoma virus. *Nature* (*Lond.*) **225,** 24 (1970).
6. L. Montagnier and I. Macpherson, Croissance sélective en gélose de cellules de hamster transformées par le virus polyome. *C.R. Acad. Sci.* (*Paris*) **258,** D, 4171 (1964).
7. L. Montagnier, R. Vigier, J. Boué and A. Boué, Transformation en milieu gélifié d'une souche diploide de cellules embryonnaires humaines par le virus de Rous (souche de Schmidt-Ruppin). *C. R. Acad. Sci.* (*Paris*) **265** D, 2161 (1967).
8. G. Deysson, Antimitotic substances. In *International Review of Cytology*, (Edited by G. H. Bourne and J. F. Danielli) **24,** 99. Academic Press, New York. (1968).
9. R. Oftebro, Ø. Grimmer, T. B. Øyen, S. G. Laland, 5-fluoropyrimidin-2-one, a new metaphase arresting agent. *Biochem. Pharmacol.* **21,** 245, (1972).

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A Pilot Study for the Evaluation of a New Regime of Combined Therapy for the Radical Treatment of Marginally Operable Rectal (or Colo-Rectal) Cancer

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Abstract—*A pilot study for the evaluation of a combined pre-operative chemotherapy (5-FU) plus radiotherapy (3450 rads in 18 days), followed by radical surgery within 2 weeks was carried out as a new regime for the radical cure of borderline operability rectal (or colo-rectal) cancer. Twenty-five patients were included in the study. They showed good tolerance to the pre-operative combined therapy, with minimal, or no side effects and hardly any increase in post-operative complications. There were 3 post-operative mortalities (12%); 2 cases were found to be inoperable (8%). Of the remaining 20 patients who completed the planned regime, 15 are free of any disease for a period varying from 15 to 25 months (75%); 2 cases alive but with local recurrences in the perineal sinuses (10%), while 3 patients died of unrelated causes (15%), with no evidence of malignancy at post mortem.*

INTRODUCTION

DUTCH literature and statistics show that cancer of the rectum is the seventh most common malignant tumour in the Netherlands. It has an overall crude survival rate of about 21% and a corrected survival rate of about 25% [1]. This rate shows little or no change over the past 40 years despite modern advances in surgical and anaesthetic techniques. This clearly indicates that a critical look at our current methods of therapy is overdue.

MATERIAL AND METHODS

Since the prognosis of rectal cancer is closely related to the stage of the primary lesion, and is decidedly worse in cases with lymph node metastases at the time of operation (i.e. Duke's C cases), it was decided that in this study only patients with locally moderately advanced disease—mainly $T_3N_xM_0$—(ranging from late T_2 to early T_4) should be included. Some locally recurrent, but operable cases were also included.

The method used was a preoperative combined course of chemotherapy plus radiotherapy, followed as soon as possible (within the first 2 weeks after irradiation) by radical surgery.

Treatment techniques

Chemotherapy. 5-Fluoro-uracil was given intravenously with an infusion of saline over 10 min. The daily dose was 10 mg/kg body weight, given in the morning and repeated for 4 days only (Monday till Thursday) while radiotherapy was given in the afternoons

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Radiotherapy. This was given on 6 MeV linear accelerator via two parallel opposing fields—anterior and posterior. The field was termed the Banjo field, or diamond and chimney field It extended from the lower border of the obturator foramina inferiorly (or lower if necessary) to the upper border of the second lumbar vertebra superiorly It thus covered both the primary tumour and its immediate lymph node drainage (Fig. 1).

One case of carcinoma of the descending colon was treated by two parallel opposing L-shaped fields. The horizontal limb covered the primary tumour while the vertical limb covered the para-aortic nodes.

Dosage. The tumour dose was 230 rads/fraction. Treatment was given daily in the afternoon 5 days a week for 3 weeks (i.e. 15 fractions) delivering a total dose of 3450 rads in 18 days.

Surgery. Radical surgery followed within 2 weeks after radiotherapy.

Patients

The total number of patients admitted into the study was 25, of whom 9 were females and 16 were males. The youngest patient was a woman 41 years of age, while the oldest patient was a man 82 years of age; the average age being 61 years. The first patient was admitted on 31 August, 1971, while the last patient was admitted on 6 June, 1972.

This analysis was carried out during the month of September 1973, thus allowing for a period of follow-up varying from 15 to 25 months.

All cases were histologically proven adenocarcinomas—of varying degrees of differentiation—24 of the rectum and one of the descending colon. Twenty-two cases were primary tumours and their staging according to the TNM-classification was as follows (Table 1):

Table 1. *Distribution of cases according to TNM-classification*

Late	$T_2N_xM_0$	11
	$T_3N_xM_0$	6
Early	$T_4N_xM_0$	5
Operable local recurrence		3
Total		25

11 cases were late $T_2N_xM_0$; 6 cases were $T_3N_xM_0$, and 5 cases were early $T_4N_xM_0$. The remaining 3 cases were locally recurrent, but operable tumours. For their pre-operative management, all patients were admitted into the Rotterdam Radiotherapy Institute—at least for the first week of their treatment—during which time 5-fluoro-uracil was adminstered intravenously in the morning (about 10.00 hours) for 4 successive days from Monday until Thursday inclusive, while radiotherapy was given in the afternoon (about 16.00 hours). A blood count was performed every morning during this week to ensure that the haemopoietic system was able to tolerate the combined therapy.

Patients who lived near the Institute were allowed to go home after the first week and to attend the remainder of their radiation treatment as out-patients, while the rest remained in-patients.

Blood counts were done 2 or 3 times weekly during the remaining period of radiotherapy.

RESULTS

Side effects of this therapy were invariably minimal and none of the patients had to have their treatments interrupted because of any severe reactions (Table 2).

Gastro-intestinal disturbances were mild; 10 cases of diarrhoea, which was satisfactorily controlled by tanna albuminum tablets, one case of nausea which responded well to Vit. B_6 treatment, and no cases of vomiting.

Table 2. *Side effects*

Diarrhoea	10
Nausea	1
Vomiting	0
Leucopenia	0
Thrombopenia	4

Haemopoietic depression was minimal. Generally there was a depression—followed by a rapid recovery—in leucocyte and platelet counts by the end of the second week or early in the third week. None of the patients had a white cell count below 2500 counts/mm^3. One patient had thrombocytopenia below 50,000 counts/mm^3 and 3 had thrombocytopenia below 100,000 counts/mm^3.

Surgery took place within the next 2 weeks after radiotherapy with an average of 10 days delay. Nineteen patients had abdomino-perineal resection of the rectum; 4 patients had radical resection with end-to-end anastomosis

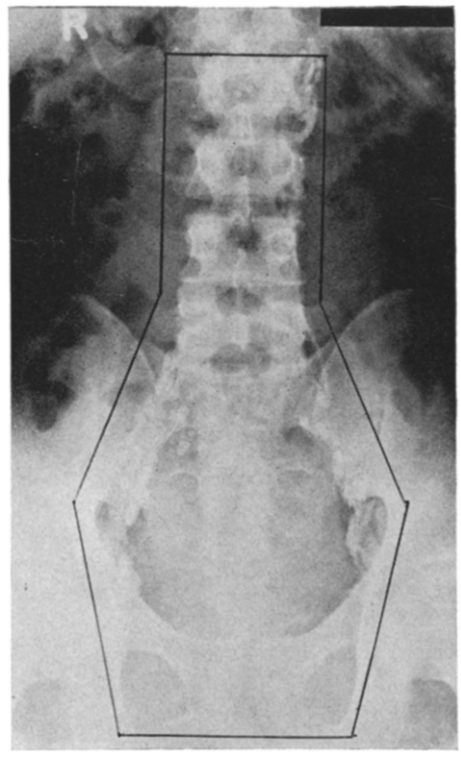


Fig. 1. *Two parallel opposing fields of irradiation*

and 2 patients were found to be inoperable—one due to bladder involvement and the other due to liver metastases (Table 3). The operative

Table 3. Type of operation

Abdomino-perineal	19	76%
Resection + anastomosis	4	16%
Inoperable	2	8%
Total	25	100%

procedure was described as easy in 15 cases (60%) and only moderately difficult due to adhesions in 7 cases (28%). It was difficult in one case because of extreme obesity (4%) and inoperable in 2 cases (8%), (Table 4). Post-operative course was uneventful in 16 cases (64%). In 7 cases (28%) there was wound infection with or without sinus formation—mainly in the perineal wound—and in 2 cases (8%) there was paralytic ileus (Table 5). There were 3 cases of post-operative death (12%)—2 were due to paralytic ileus (see above) and one was due to pulmonary embolism.

Table 4. Operative procedure

Easy	15	60%
Moderately difficult (adhesions)	7	28%
Difficult (obesity)	1	4%
Inoperable	2	8%
Total	25	100%

Table 5. Post-operative course

Uneventful	16	64%
Wound infection ± sinus formation	7	28%
Paralytic ileus	2	8%
Total	25	100%

The surgical specimens of the operated 23 cases were examined histologically for the presence, or absence of viable tumour cells at the primary site, as well as for positive lymph nodes. The tumour site showed no signs of malignant cells in 4 cases (17·4%), only necrotic or degenerate tumour cells in 6 cases (26·1%) and viable tumour cells in 13 cases (56·5%), (Table 6). Lymph node metastases

Table 6. Histology of tumour site (23 cases)

No tumour cells	4	17·4%
Necrotic or degenerate tumour cells	6	26·1%
Viable tumour cells	13	56·5%

were found in 3 cases only (13%). Liver metastases were found in one case only (4·3%). Since 3 patients died post-operatively and 2 patients were found to be inoperable, 20 patients who completed the planned regime were available for follow-up assessment for a varying period from 15 to 25 months (Table 7). Of these, there are 15 patients who are well with no evidence of disease (75%); 2 patients who have local recurrences in persistent perineal sinuses (10%) and 3 patients who died of unrelated causes (15%). Post mortem examination of all 3 showed no evidence of malignancy, but nevertheless they are counted as failures, otherwise the disease-free patients would have amounted to 90%.

Table 7. Follow-up record (20 cases)

Alive, no evidence of disease	15	75%
Alive with local recurrence	2	10%
Dead of unrelated cause	3	15%

DISCUSSION

This pilot study is too small to allow for accurate conclusions to be drawn. But it rings a note of cautious optimism as to the use of combined therapy for the management of moderately advanced cancer of the rectum. This is demonstrated well in the examination of the surgical specimens.

Macroscopically there was invariably a great reduction in the tumour size, thus rendering at least 4 out of 5 $T_4N_xM_0$ (80%) of doubtful operability cases into the operable category. Histologically, there was no evidence of tumour cells in 4 tumour sites (17·4%) and in 6 further cases (26·1%) the site was necrotic with degenerate

cells. The greatest achievement lies in the reduction of lymph node metastases, which features in 60–70% of cases of rectal tumours of this stage, but in this series only 3 cases (13%) were found to have positive nodes.

Despite the complexity of this policy, side effects and surgical complications were minimal and should form no actual hazard for the routine application of such a regime once its superiority has been confirmed through well controlled clinical trials.

A great deal of major surgical procedures which are performed for the radical cure of colo-rectal cancer fail to do so. The main causes of failure are:

1. local recurrence at the primary site,
2. lymph node metastasis,
3. haematogenous spread to distant organs, especially the liver, lungs, bones, brain, etc,
4. implantation nodules in the operation scar,
5. spill into the peritoneal cavity.

All these causes can be attributed to "viable tumour cells" at one or more of the following sites:

1. the primary site,
2. in the lymphatic circulation,
3. in the blood stream,
4. as microscopic metastatic foci in distant organs.

Therefore, the answer to our problem is to render the tumour cells, regardless of locality, incapable of propagation. To do this, we have to deal with the mass of the primary tumour (i.e. local treatment), as well as with the "floating cells" in the circulation together with the "foci" in distant organs (i.e. systemic treatment). In other words, a combined therapeutic approach.

Our present day treatment modalities are either "local" in the form of surgery or radiotherapy, or "general," i.e. systemic in the form of chemotherapy. Of the most promising combinations so far attempted, is the use of pre-operative irradiation of the primary tumour site and its immediate lymphatic drainage [2–7].

Hoye and Smith [8] administered radiation in mice with different types of tumours 24 hr before surgery in doses sufficient to stop growth, or cause regression of the primary tumour; the tumour cells' ability to implant and grow was reduced by 90 per cent. One explanation may be that the effect of the irradiation is to inhibit the proclivity of the cancer cells, disseminated at the time of surgery, to grow. Pre-operative radiotherapy gained a lot of popularity in recent years. To summarise its merits we can mention the following:

(a) reduction in the tumour size and vascularity,

(b) rendering some inoperable lesions operable,

(c) diminishing the incidence of local recurrence,

(d) diminishing the incidence of distant metastases, both spontaneous and as a result of surgical manipulations.

In 1958, Heidelberger and associates demonstrated that regression of certain transplanted animal tumours, as well as animal survival, were enhanced by the addition of 5-FU to radiation therapy. Moertel and co-workers [9] initiated their studies in this field in 1961 on advanced gastro-intestinal tumours and came to the following conclusions: combined 5-FU and supervoltage radiotherapy may provide significant objective and symptomatic palliation for some patients with advanced gastro-intestinal cancer. Thirty-two patients with colo-rectal cancer were given 5-FU plus radiotherapy, and 33 similar patients were given placebo plus radiotherapy. The mean duration of the symptomatic control was only 11·5 months in the placebo group, compared with 17 months in the group given 5-FU which is statistically significant.

Since chemotherapy plus radiotherapy have synergistic effects it was decided to give them both in slightly reduced doses hence the 10 mg/kg body weight $\times 4$ of 5-FU instead of the full loading dose of 15 mg/kg body weight $\times 5$. The irradiation dose of 3450 rads in 18 days is roughly equivalent biologically to 4000 rads in 4 weeks, and the effect is further enhanced through the addition of 5-FU. A shorter overall treatment time has been chosen in order to obviate the risk of higher incidence of liver metastases through the prolongation of time-lag between clinical diagnosis and actual surgery.

It is only logical to expect a higher beneficial effect from such a pre-operative combination, both locally on the primary site with its lymphatic drainage and systemically on the "floating cells" in the circulation and the distant metastatic "foci". The radical surgery which will follow will have a greater chance of success. There is a feeling of optimism about the superiority of the regime, which has to be confirmed via randomised clinical trials, which have already been organized [10].

REFERENCES

1. L. MEINSMA, Vijfjaars overlevingscijfers no kankerbehandeling. *Diss Amsterdam* (1963).
2. C. V. ALLEN and W. S. FLETCHER, Observations on preoperative irradiation of rectosigmoid carcinoma. *Amer. J. Roentgenol.* **108,** 136 (1970).
3. C. V. ALLEN and W. S. FLETCHER, A pilot study on preoperative irradiation of rectosigmoid carcinoma. *Amer. J. Roentgenol.* **114,** 504 (1972).
4. R. W. DWIGHT, G. A. HIGGINS, *et al.*, Preoperative radiation and surgery for carcinoma of the sigmoid colon and rectum. *Amer. J. Surg.* **123,** 93 (1972).
5. W. S. FLETCHER and C. V. ALLEN, Preoperative irradiation for carcinoma of the colon and rectum. *Amer. J. Surg.* **109,** 76 (1965).
6. M. M. KLIGERMAN, N. URDANETA, A. KNOWLTON *et al.*, Preoperative irradiation of rectumsigmoid carcinoma including its regional lymph nodes. *Amer. J. Roentgenol.* **114,** 498 (1972).
7. R. H. LEAMING, M. W. STEARNS and M. R. DEDDISH, Preoperative irradiation in rectal carcinoma. *Radiology* **77,** 257 (1961).
8. C. H. HOYE and R. R. SMITH, The effectiveness of small amounts of preoperative irradiation in preventing the growth of tumour cells disseminated at surgery. *Cancer,* **14,** 284 (1961).
9. C. G. MOERTEL and R. J. REITEMEIR, Advanced gastro-intestinal cancer. In *Clinical Management and Chemotherapy.* Harper & Row, New York.
10. European Organization for Research on Treatment of Cancer (E.O.R.T.C.)—Gastro-intestinal Group. Clinical Trial. (Private communication—Protocol and early results are being prepared for publication).

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Damage to Mouse Lung With Neutrons and X-Rays

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Abstract— *Mice die of radiation pneumonitis between* 80 *and* 180 *days after irradiation of the thorax. Using the endpoint of* LD_{50}, *the RBE for fast neutrons has been measured and shown to be low compared to most other normal tissues. The effects of fractionation of X-rays are similar to those found in other tissues. A maximum sparing, obtained by measurement of* D_2–D_1, *was found to be about* 500 *rads of X-rays at an interval of* 6 *hr between doses. It has been tentatively attributed to repair of sublethal damage of surviving cells. For time intervals greater than* 6 *hr between doses, the* LD_{50} *is increased by about* 8 *rads per day up to* 28 *days. This increase is attributed to a slow repopulation of surviving cells.*

INTRODUCTION

THE RELATIVE biological effectiveness (RBE) of fast neutrons for mammalian tissues varies with the dose of radiation per fraction [1, 2] and also varies from tissue to tissue [3]. Skin has been the subject of the greatest number of investigations, and RBE values for 4 species have been measured over a wide range of doses and doses per fraction, viz. man, pig, rat and mouse. When RBE is plotted as a function of dose per fraction, the data from all 4 species lie on the same smooth curve [4]. Thus, for skin it is possible to extrapolate RBE measurements from animals to man with confidence. Similar information is not yet available for other tissues, but the observations on skin do justify the use of small animals to investigate RBE as a function of dose per fraction for a variety of tissues and the cautious extrapolation of the RBE values obtained to man.

The present communication describes experiments involving damage to mouse lung caused by irradiation with X-rays or fast neutrons. The damage is similar to that observed in patients suffering from late radiation pneumonitis. If both lungs are irradiated it results in the death of the animal within 6 months [5]. The effects of both single doses and 2 fractions are described taking survival at 180 days as the endpoint of the experiment. This system is likely to be of special interest to the radiotherapist because: (a) the damage observed is an example of later adiation damage, (b) it seems likely that the damage is largely vascular and (c) damage to normal lung tissue may be a limiting factor in radiotherapy.

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MATERIAL AND METHODS

The thorax of each mouse was irradiated with either X-rays or fast neutrons, while the rest of the animal was shielded. Female TO mice, 12–15 weeks old, were used, and were anaesthetised with 60 mg/kg of sodium pentobarbitone (Nembutal) before each irradiation. The mice were irradiated at room temperature while breathing air.

The X-rays used were generated by a 250 kVp X-ray set, filtered by 0·25 mm Cu and 1 mm Al and having a HVL of 1·3 mm Cu. The dose rate was 170 rads/min. The beam was directed vertically downwards, with 6 mice irradiated simultaneously, each lying face upwards in an individual perspex holder. The

thorax was exposed through a window 2·5 × 3·0 cm, with both the head and the abdomen below the diaphragm, which was located by the xiphisternum, shielded by lead. The 6 mice were arranged radially and rotated during irradiation to ensure a uniform dose distribution.

The neutrons were produced by bombarding a beryllium target with 16 MeV deuterons from the Medical Research Council's cyclotron at Hammersmith. The mean neutron energy was about 7·5 MeV [6], and the gamma ray contamination was about 3% [7]. The beam was collimated by means of paraffin wax and finally by steel to give identical field sizes to those used for the X-irradiation. The beam was horizontal and 8 mice were irradiated together. As it was impractical to rotate the collimator, there was a dose variation from mouse to mouse round the field but amounting to less than ±5% of the mean value and this was considered acceptable. The whole body dose was negligible with X-rays, but with neutrons it was about 10% of the dose given to the thorax. Each mouse had its thorax covered with a thin sheet of lead, so that the secondary protons produced in the perspex jig were stopped and some skin sparing was obtained. Full build up is obtained in 1 mm of tissue, so only the dose to skin was reduced. This technique was developed for use in neutron therapy [8].

RESULTS

After irradiation of the thorax, mice surviving 40 days die at about day 80, (Fig. 1). This was first observed by Phillips, [5] who suggested that the mice were dying of pulmonary damage (see Discussion). Mice surviving 180 days die at a rate similar to that of unirradiated controls for at least the next four months. The pattern of death was the same after irradiation with fast neutrons as with X-rays (Fig. 1). To construct dose-effect curves, the endpoint chosen was the fraction of mice that died between days 40 and 180. With X-rays, each dose point was derived from irradiation of 18 mice, and with neutrons, from 16 mice. Typically, about 100 mice were used to derive each dose-effect curve. From the data, the LD_{50} values, 40–180 days and 95% confidence limits were calculated from probit analysis (Table 1).

Dose-effect curves for single doses and 2 fractions of X-rays, separated by various time intervals are shown in Fig. 2. For clarity, the calculated LD_{50} values with 95% confidence limits are shown for 2 curves only. All the data shown in Fig. 2 were obtained from a single experiment. With increasing time between fractions, additional dose was required, but the most dramatic change occurred within the first 2 hr. For any level of survival, mice irradiated with 2 doses separated by 2 hr require a dose of about 400 rads of X-rays greater than the single dose. The additional dose required in 2 fractions, to produce the same level of damage as a single dose may be designated D_2–D_1 [9]. D_2–D_1 is plotted as a function of time between fractions in hr (Fig. 3) and days (Fig. 4). Figure 3 shows that a maximum value for D_2–D_1 of about 500 rads was reached at about 6 hr between fractions. Over a longer period of time extending to 28 days, D_2–D_1 increased at the slower rate of about 8 rads/day.

Dose-effect curves for single doses and 2 fractions, separated by 24 hr are shown in Fig. 5 for both X-rays and fast neutrons. These

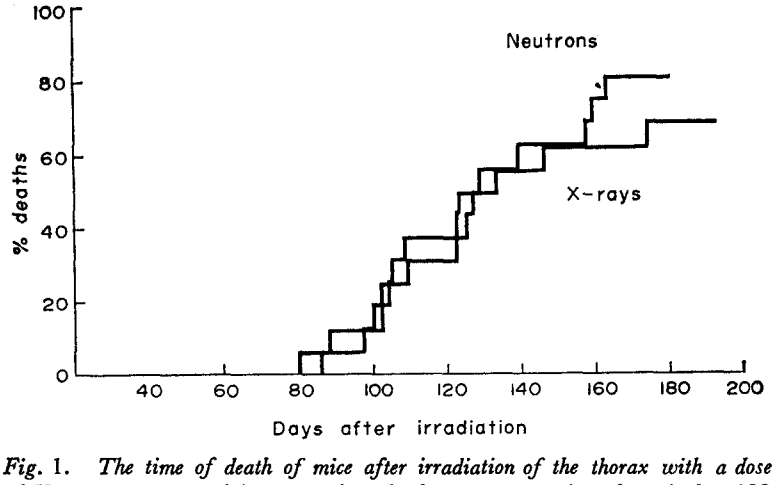


Fig. 1. The time of death of mice after irradiation of the thorax with a dose of X-rays or neutrons giving approximately the same proportion of survival at 180 days post-irradiation.

Table 1. LD_{50}/180 *doses with* 95% *confidence limits after irradiation of the thorax of mice. Mice irradiated with* 250 *kVp X-rays or fast neutrons*

	X-rays Experiment 1	X-rays Experiment 2	X-rays Experiments 1+2	Neutrons
Single dose	1078+1131 −1025	1234+1335 −1172	1166+1287 −1083	778+818 −741
2 Fractions separated by				
2 hr	1467+1528 −1376			
6 hr	1606+1926 −1214			
24 hr	1454+1520 −1386	1539+1607 −1499	1515+1556 −1481	968+103 −905
9 days	1690+1762 −1619			
28 days	1834+1931 −1739			

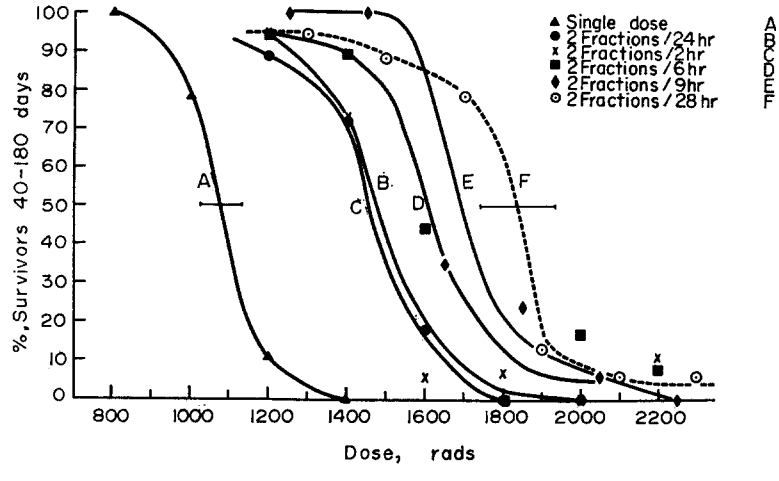


Fig. 2. *Dose–effect curves for mouse survival at* 180 *days after irradiation of the thorax with single doses of X-rays (curve A) or* 2 *equal doses of X-rays separated by* 24 *hr(B),* 2 *hr (C),* 6 *hr (D),* 9 *days (E) and* 28 *days (F).*

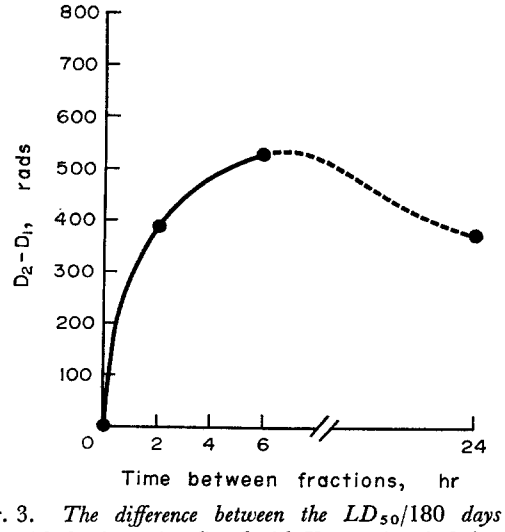


Fig. 3. *The difference between the* LD_{50}/180 *days for mice with the thorax irradiated with X-rays given in* 2 *fractions or a single dose* (D_2–D_1) *as a function of time between fractions up to* 24 *hr.*

data were derived from several experiments, and since the data from each were not significantly different, the results were pooled. The RBE is defined as the ratio of dose of X-rays to that of neutrons to produce a given level of injury (in this case LD_{50} 40–180 days). The RBE for single doses was 1·5 at the LD_{50} value of 780 rads of neutrons and for 2 fractions the RBE was 1·6 at the LD_{50} value of 485 rads of neutrons per fraction. The D_2–D_1 value at 24 hr was approximately 400 rads of X-rays, and approximately 200 rads of neutrons.

DISCUSSION

Histological examination of mice dying between 80 and 180 days after irradiation of the thorax by either X-rays or neutrons showed

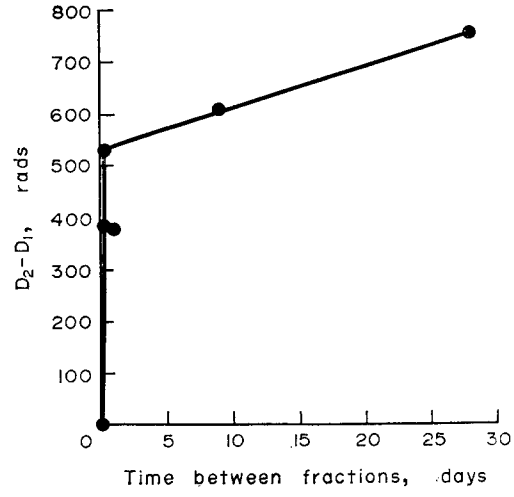


Fig. 4. The difference between the LD_{50}/180 days for mice with the thorax irradiated with X-rays given in 2 fractions or a single dose (D_2–D_1) as a function of time between fractions up to 28 days.

the lungs to be highly cellular and spotted with areas of consolidation. The septal walls were thickened and the alveolar space reduced, often containing large cells epithelial in appearance. At this time there was no evidence of oesophageal damage although after the highest doses used in our experiments, some mice died before day 30 post irradiation, with perforation of the oesophagus (Hornsey and Field, in preparation). This has also been observed by Phillips and Margolis [5] and Kurohara and Cassarett [10]. In the present experiments, mice that survived for less than 40 days post irradiation were excluded from the calculations.

The primary lesion in the lung is not known with certainty. Kurohara and Cassarett [9] considered that, in rats, death after irradiation of the thorax was due to pneumonic infection superimposed on developing pulmonary fibrosis. From electron microscope studies of irradiated lung, Phillips [11] concluded that the primary mode of damage was the killing of endothelial cells which led to capillary blockage followed by plasma cell, mast cell and fibroblast infiltration. Maisin [12], from a study of radiation pneumonitis in mice concluded that the damage was due to parenchymal cell death caused both by the direct effects of radiation on the parenchymal cells, and indirect damage secondary to radiation injury of the vascular connective tissue.

It was seen that fractionation of X-rays had a tissue-sparing effect which was greater at 6 hr than at 2 hr (Fig. 3). If the lung damage which results in the death of the mice is caused primarily by radiation damage to cells, as suggested by both Phillips and Maisin, then the sparing effect of fractionated radiation may be due to accumulation and subsequent repair of sublethal damage [13]. The value of D_2–D_1 obtained (about 500 rads at 6 hr) is similar to values obtained for other normal tissues, e.g. skin and gut, where the damage is known to be caused by the loss of reproductive integrity of the respective stem cells.

If tissues are repopulated in the interval between dose fractions, this will result in an increase in LD_{50} with time between fractions. For the lung, this increase after the first few hours amounts to approximately 8 rads per day, whereas for skin, the corresponding value is about 30 rads per day [14–17]. This difference between lung and skin may be due to differences in the sensitivity of the critical cell populations or to differences in the cell renewal times. We do not know which is the critical

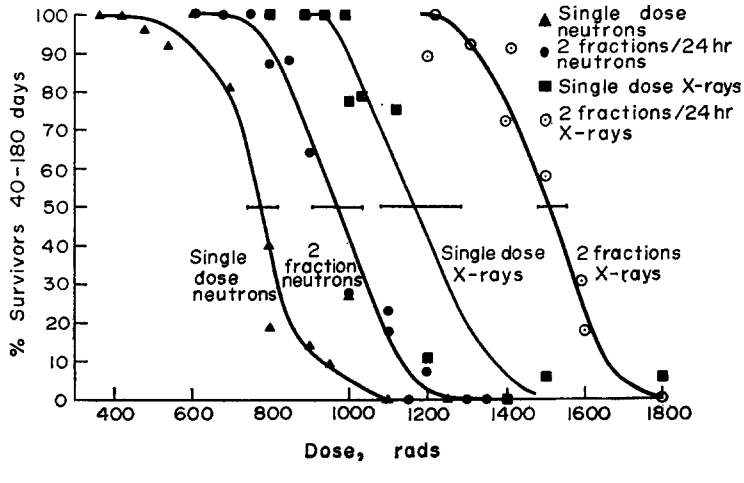


Fig. 5. The percentage of mice surviving at 180 days after irradiation of the thorax with single doses or two equal doses separated by 24 hr of 250 kVp X-rays or fast neutrons.

cell population responsible for lung damage but evidence has been sited, [11, 12] which suggests that the capillary endothelial cells are involved. The sensitivity of capillary endothelial cells has been estimated by Hopewell and Patterson [18], from observations on the maintenance of capillary loops in pig skin, and by Reinhold [19] in rat skin, at a D_0 of about 1000 rads. If the sensitivity of lung capillary endothelial cells is similar, then the 8 rad/day increase in D_2–D_1 would imply a cell doubling time of about 100 days. Tannock, [20] made estimates of the probable doubling times of endothelial cells in a number of unstimulated adult murine tissues to be 2 months or more. Reinhold and Buisman [21], from the numbers of developing capillary buds estimated a D_0 for capillary endothelial cells of 170 rads but this was for endothelium which had been stimulated to grow and is probably not relevant to the adult unstimulated lung endothelium, to which our repopulation value of about 8 rads/day probably applies. After irradiation, some stimulation induced by the radiation killed cells may ultimately occur. Hoassler [22] found no increase in proliferation of the capillary endothelial cells in mouse brain for at least 40 days after irradiation while Fajardo and Stewart [23] found an increase in the proliferative activity of the myocardial capillary endothelium at 40–70 days after irradiation. Such stimulation may initially lead to an increase in cell death as more damaged endothelial cells go into division. If the time scale in the lung capillary endothelium is similar to that of the myocardial endothelium, then such stimulation precedes, and may contribute to, the death of the animal.

Postmortem examinations, both macroscopic and microscopic, showed no qualitative differences in the response of mouse lung to X-rays or neutrons. The death rate after X-rays or neutrons was the same after doses giving the same proportion of survivors at 180 days (Fig. 1). Many other tissues, e.g. skin, gut, and some experimental tumours also show no qualitative differences in response [4]. Only in these circumstances is it valid to use dose–effect curves to derive values of RBE.

The RBE values obtained for single doses and 2 fractions separated by 24 hr were 1·5 and 1·6 respectively. Comparison of these values with those for other tissues at about the same neutron dose shows RBE values for lung damage about 40% less than for intestinal damage and about 30% less than for skin damage [4, 24], but similar to values for damage to the haemopoietic system [2]. After irradiation with fast neutrons D_2–D_1 at 24 hr was approximately 200 rad, i.e. about 1/4 of the single dose LD_{50} value, while after X-rays, the D_2–D_1 was approximately 700 rad, i.e. about 1/3 of the single dose LD_{50} value with X rays. If the lung damage we have observed is the result of cell killing, then the D_2–D_1 values will reflect repair of sublethal damage. There is clearly a considerable amount of repair after both X-rays and neutrons and the proportion of repair after neutrons, compared with X-rays, is greater than that seen in either skin [25–27] or gut [28, 29]. Where there is relatively little change in the amount of repair of sublethal damage after neutrons compared with X-rays then the RBE will more closely reflect changes in D_0. The low RBE obtained for the haemopoietic tissue also largely reflects changes in D_0, for there is relatively little repair after X-rays or neutrons [30].

The low RBE for late lung damage makes it particularly important to know which cells are critical in determining the gross response of the tissue. If, for example, it is those of the capillary endothelium, will they show the same sensitivity in other tissues? If so, then the low RBE values might also apply to late effects in other tissues. In radiotherapy the late effects are of particular concern.

The results presented in this paper do imply a clear therapeutic advantage for neutrons where late lung damage is the limiting factor providing: (a) human lung responds like mouse lung, (b) the low RBE values are maintained at doses per fraction which are within the therapeutic range, (c) the tumour does not have an equally low RBE [31].

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REFERENCES

1. J. F. Fowler and R. L. Morgan, Pretherapeutic experiments with the fast neutron beam from the M.R.C. Cyclotron. VIII. General review. *Brit. J. Radiol.* **36,** 115 (1963).
2. S. B. Field, The relative biological effectiveness of fast neutrons for mammalian tissues. *Radiology* **93,** 915 (1969).

3. S. Hornsey, The relative biological effectiveness of fast neutrons for intestinal damage. *Radiology* **97,** 649 (1970).
4. 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).
5. T. L. Phillips and L. Margolis, Radiation pathology and the clinical response of lung and esophagus. In *Frontiers of Radiation Therapy and Oncology.* (Edited by J. M. Vaeth) Vol. 6, p. 254 S. Karger, Basel (1972).
6. C. J. Parnell, A fast neutron spectrometer and its use in determining the energy spectra of some cyclotron-produced fast neutron beams. *Brit. J. Radiol.* **45,** 452 (1972).
7. D. K. Bewley and C. J. Parnell, The fast neutron beam from the M.R.C. cyclotron. *Brit. J. Radiol.* **42,** 281 (1969).
8. M. Catterall, C. Rogers, R. H. Thomlinson and S. B. Field, An investigation into the clinical effects of fast neutrons. *Brit. J. Radiol.* **44,** 603 (1971).
9. J. F. Fowler, Kinetics of injury and repair to mammalian tissue by high-LET radiation. *Radiat. Res. Suppl.* **7,** 276 (1967).
10. S. S. Kurohara and G. W. Cassarett, Effects of single thoracic X-ray exposure in rats. *Radiat. Res.* **52,** 263 (1972).
11. T. L. Phillips, An ultrastructural study of the development of radiation injury in the lung. *Radiology* **87,** 49 (1966).
12. J. R. Maisin, The ultrastructure of the lung of mice exposed to a supralethal dose of ionizing radiation on the thorax. *Radiat. Res.* **44,** 545 (1970).
13. M. M. Elkind and H. Sutton, X-ray damage and recovery in mammalian cells grown in culture. *Nature (Lond.)* **184,** 1293 (1959).
14. 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).
15. 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. *Radiat. Res.* **41,** 450 (1970).
16. H. R. Withers, The dose–survival relationship for irradiation of epithelial cells of mouse skin. *Brit. J. Radiol.* **40,** 187 (1967).
17. L. Cohen and M. J. Scott, Fractionation procedures in radiation therapy: a computerized approach to evaluation. *Brit. J. Radiol.* **41,** 529 (1968).
18. J. W. Hopewell and T. J. S. Patterson, The effects of previous X-irradiation on the revascularisation of free skin grafts in the pig. *Proc. British Microcirculat. Society*, Northampton, 1971.
19. H. S. Reinhold, Radiation and the microcirculation, In *Frontiers of Radiation Therapy and Oncology.* (Edited by J. M. Vaeth), Vol. 6 p. 44. S. Karger, Basel (1972).
20. I. F. Tannock and S. Hayashi, The proliferation of capillary endothelial cells. *Cancer Res.* **32,** 77 (1972).
21. H. S. Reinhold and G. H. Buisman, Radiosensitivity of capillary endothelium. *Brit. J. Radiol.* **46,** 54 (1972).
22. O. Hoassler, Incorporation of tritiated thymidine into mouse brain after single doses of X-rays. An autoradiographic study. *J. Neuropath. exp. Neurol.* **25,** 97 (1966).
23. L. F. Fajardo and J. R. Stewart, Capillary injury preceding radiation-induced myocardial fibrosis. *Radiology* **101,** 429 (1971).
24. S. Hornsey and S. B. Field, The RBE for cyclotron neutrons for effects on normal tissues. *Europ. J. Cancer* **10,** 231 (1974).
25. 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. *Radiat. Res.* **29,** 71 (1966).
26. J. Denekamp and J. F. Fowler, Further investigations of the response of irradiated mouse skin. *Inter. J. Radiobiol.* **10,** 435 (1966).
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. Part II Fractionation; recovery and reoxygenation. *Brit. J. Radiol.* **41,** 597 (1968).
28. 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).

29. S. HORNSEY, The effectiveness of fast neutrons compared with low LET radiation on cell survival measured in the mouse jejunum. *Radiat. Res.* **55,** 58 (1973).
30. G. SILINI and Y. MARUYAMA, X-ray and fast neutron survival response of 5-bromo-deoxycytidine treated bone-marrow cells. *Int. J. Radiat. Biol.* **9,** 605 (1965).
31. J. F. HOWLETT, R. H. THOMLINSON and T. ALPER, A marked dependence of the comparative effectiveness of neutrons on tumour line, and its implications for clinical trials. *Brit. J. Radiol.* (In press).

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Letter to the Editor

A Comparison of the Phosphofructokinase Isoenzyme Profiles of Transplantable Hepatomas and Normal Liver*

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WE HAVE shown previously that it is possible to distinguish at least nine forms of phosphofructokinase (PFK: ATP:D-fructose-6-phosphate 1-phosphoransferase EC 2.7.1.11) in rat tissues using DEAE-cellulose chromatography, the liver apparently containing five of these forms [1]. Two major groups of liver isoenzymes, designated L1 and L2 were separated and shown to have different kinetic properties, these properties being different again from those of rat muscle PFK [2]. Other authors, while reporting that liver PFK is homogeneous, find that transplantable hepatomas contain more than one isoenzyme [3, 4]. The implication is that forms of PFK not present in normal liver occur in hepatomas. In this paper we re-examine this question in the light of our new knowledge of the heterogeneity of liver PFK, using a series of four hepatoma lines having widely differing growth rates and states of differentiation.

The hepatoma material used was from the Morris hepatomas 9618A (highly differentiated), 16 (well differentiated) and 7777 (poorly differentiated) or Novikoff ascites hepatoma cells (very poorly differentiated). Liver and tumour tissue supernatants, in 50 mM Tris–HCl buffer pH 8·0 containing 10 mM KF and stabilizers [1, 2], were obtained by the centrifugation, at 150,000g for 30 min, of tissue homogenates or French press disrupted ascites cells. DEAE–cellulose chromatography was performed as previously described [1, 2].

The total PFK activity was similar in liver (2·8 U/g wet wt) and tumour 9618A (2·7 U/g), whereas the less well differentiated tumours 16 (4·5 U/g), 7777 (7·8 U/g) and Novikoff cells (23 U/ml packed cells) had progressively higher activities. These results are the means of at least four observations except the value for 9618A which is the mean of three. There would appear to be increasing total PFK activity with increasing growth rate.

The comparison of the PFK isoenzyme profiles for liver and the tumours can be seen in Fig. 1. All the tumours show differences in isoenzyme composition from that of the rat liver but the changes are within the range of isoenzyme types found in the liver. Moreover there is no obvious correlation between the change in the isoenzyme pattern and the degree of differentiation, the best differentiated and least well differentiated cells, 9618A and Novikoff respectively, showing the greatest deviation from the normal liver profile. We find, therefore, no evidence of new forms of PFK arising in tumours.

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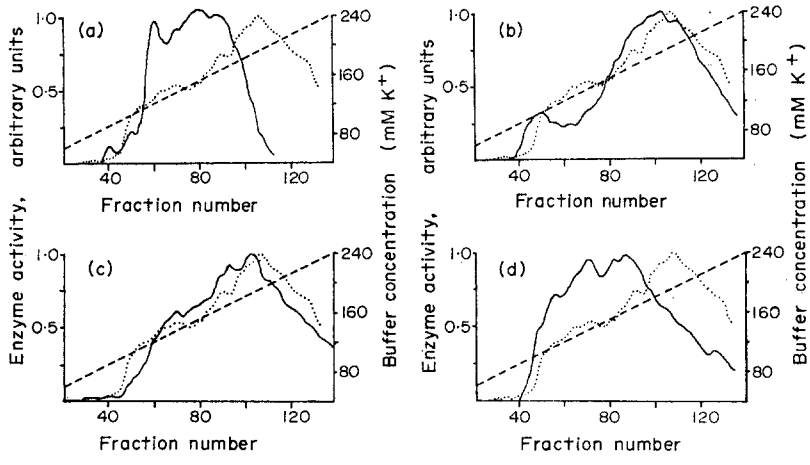


Fig. 1. *Gradient elution from DEAE-cellulose of the phospho-fructokinase of normal rat liver and transplantable hepatomas.* (a) *Hepatoma* 9618*A;* (b) *Hepatoma* 16*;* (c) *Hepatoma* 7777*;* (d) *Novikoff Ascites Hepatoma. In all four diagrams the liver PFK elution profile* (. . . . .) *is shown compared with the hepatoma elution profile* (——). *All chromatographic runs were carried out under similar conditions with elution by a linear gradient from* 100 *mM to* 630 *mM Tris–potassium phosphate buffer, pH* 8·0 [1].

Equivalent amounts of PFK in the supernatant fluids from tumours and whole liver were allowed to react with excess antiserum, raised against rat muscle PFK [1], and the residual activity determined after centrifugation. In no case was a greater proportion of the PFK activity precipitated from the tumour supernatant than from the liver supernatant. This is consistent with our chromatographic results indicating that no muscle-type enzymes [1] are produced by the tumours.

Our results show that for these hepatomas with widely differing growth rates there is no production of new forms of PFK not already present in the liver. Other authors [3, 4] using different hepatomas have reported that isoenzymes not present in liver were to be found in all the hepatomas except for the slow growing Morris hepatoma 7316A. It was also reported [4] that many of the hepatomas had lost the isoenzyme which was characteristic of liver tissue. However, in agreement with our results neither group found the muscle enzyme to be produced by hepatomas. Some of the discrepancies between our own results and previous reports may be due to failure to discern the heterogeneity of liver PFK in the earlier work either because a preliminary purification step was routinely used [3] or because of the inherently poor resolution of the method of separation.

Dunaway, Morris and Weber [5] have reported that the PFK isolated from the poorly differentiated Morris hepatoma 3924A was kinetically and immunologically indistinguishable from the PFK of liver. This is consistent with our finding that alterations in PFK isoenzyme content of rat hepatomas are essentially changes in the proportions of those forms found in the normal liver, as is probably the case for the isoenzyme composition of hexokinase and pyruvate kinase in normal and neoplastic tissues. However, unlike these two enzymes PFK does not show progressive deviation in isoenzyme profile with increasing growth rate of the tumour. Our results would seem to support the statement by Weinhouse [6] "In general, the alterations (in isoenzymes) seem to be sporadic and unpredictable and therefore suggest a disordered rather than a programmed mechanism of gene expression."

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REFERENCES

1. W. KIRBY and C. B. TAYLOR, Multiple forms of phosphofructokinase in rat tissues. *Int. J. Biochem.* **5,** 89 (1974a).
2. W. KIRBY and C. B. TAYLOR, Differences in the properties of phosphofructokinase isoenzymes of rat tissues. *Biochem. Soc. Trans.* (In press).
3. T. TANAKA, T. AN and Y. SAKAUE, Studies on multimolecular forms of phosphofructokinase in rat tissues. *J. Biochem.* (*Tokyo*) **69,** 609 (1971).
4. N. KURATA, T. MATSUSHIMA and T. SUGIMURA. Multiple forms of phosphofructokinase in rat tissues and rat tumours. *Biochem. biophys. Res. Commun.* **48,** 473 (1972).
5. G. A. DUNAWAY, JR., H. P. MORRIS and G. WEBER, Increased phosphofructokinase concentration in hepatoma 3924-A: enzymic and immunological evidence. *Life Sci.* **11,** (*Part II*) 909 (1972).
6. S. WEINHOUSE, Glycolysis, respiration and anomalous gene expression in experimental hepatomas. *Cancer Res.* **32,** 2007 (1972)

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Immuno-Fluorescence and Immuno-Electron Microscopy on EBV-Associated Membrane Antigen of a Nasopharyngeal Carcinoma Derived Lymphoblastoid Cell Line (LY-28)*

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Abstract—*The Epstein–Barr virus-induced, cell membrane antigen (MA), was localized on a nasopharyngeal carcinoma (NPC)-derived lymphoblastoid cell line by indirect immuno-fluorescence and immuno-electron microscopy after applying a Burkitt serum (Abwao), with anti-MA and anti-VCA reactivity.*

The immuno-fluorescence and immuno-electron microscopic observations indicated a dotted, patchy or segmental labelling of the surface of cells incubated with Abwao serum under conditions (4°C) minimizing redistribution of membrane-bound antibodies.

At the ultrastructural level, the most extensive surface labelling was seen in a small percentage of cells, which had characteristically altered nuclei and contained intranuclear and/or cytoplasmic viral particles. A larger number of cells showed smaller patches of membrane labelling, and in these cells no viral particles or degenerative changes were observed.

Extracellular viral particles, usually associated with the surface of cells, were more heavily labelled in preparations treated with the Abwao serum as compared to treatment with a heterologous antiserum. Some cells appeared to engage in active uptake of membrane associated virus particles by endocytosis.

INTRODUCTION

THE EPSTEIN–BARR virus, (EBV), a lymphotropic herpes virus of man, is capable of converting normal lymphocytes of the B-type that have a limited life-span *in vitro*, into potentially "immortal" lymphoblastoid cell lines, capable of continuous *in vitro* proliferation and also of malignant growth on heterotransplantation to immunosuppressed recipients. *In vivo*, it can cause infectious mononucleosis, a self-limiting lymphoproliferative disease. The consistent association of the viral genome with two malignant tumors, African Burkitt's lymphoma (BL), and nasopharyngeal carcinoma (NPC) has led to the suspicion that the virus is involved in the etiology of these two diseases, [1–8]. Cells of fresh BL

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biopsy specimens reveal a distinctive membrane antigen (MA), which is not observed in cells of normal lymph nodes or leukemias, [9, 10]. Similarly, lymphoblastoid lines established from NPC are positive by immunofluorescence test for MA-type antigens [11], but cell lines established from carcinomas of the head and neck, other than NPC, are not. The MA antigens can also be demonstrated on cells of established Burkitt or EBV associated lymphoblastoid cell lines, [12, 13]. Immunoelectronmicroscopy localized the MA antigen on the cell membrane of EBV-carrying lymphoblastoid lines and on the viral envelope, [14]. The relationship to EBV is indicated by serological evidence [15, 16], and by the prompt appearance of MA following exposure of MA negative cell lines to EBV, [13, 17, 19]. The present study on a NPC derived lymphoblastoid line is an attempt to correlate the immunoelectronmicroscopic labelling of MA with the fine structural appearance of virus and virus-induced cytopathic features of the cells.

MATERIAL AND METHODS

LY-28, a lymphoblastoid cell line derived from a nasopharyngeal carcinoma [20], was studied. This cell line is a virus producer as defined by the presence of EA, VCA and virus particles [6, 20, 21], and reveals a variable strong level of MA-reactivity. The cell line was propagated *in vitro* as described elsewhere [21], and cultures with a high MA-reactivity were selected by direct immuno-fluorescence tests [22]. Cells were treated with Abwao serum, a human serum with concordant high anti-MA, anti-EA and anti-VCA antibody titers [23], with a negative human serum (Dishom Muangola) or with a monkey anti-HeLa cell serum ("positive control") as summarized in Table 1. Cell bound antibody was demonstrated by indirect immuno-fluorescence, (IFL) and by immuno-electronmicroscopy (IEM) after applying rabbit anti-human immunoglobulin, (Ig) conjugated to fluorescein, (FITC) or ferritin, or rabbit anti-human immunoglobulin, I(gG)—anti-ferritin hybrid antibodies. All in- cubations were done at approximately 4°C. The FITC and ferritin conjugates were manufactured as previously described [24], and the preparation and properties of the hybrid antibodies have also been described in detail elsewhere, [25]. The hybrid reactivity was assayed by fluorescence microscopy after incubation with fluorescein conjugated apoferritin ("apofluorretin"), [25] and by electronmicroscopy after incubation with ferritin.

For electron microscopy, cell pellets were fixed in 3% cacodylate buffered glutaraldehyde and subsequently in 1% cacodylate buffered osmium tetroxide. After dehydration, the cells were embedded in Spurr low-viscosity epoxy medium, [26]. Sections were made on a Reichert OMU ultramicrotome stained by uranyl acetate and lead citrate and studied and photographed in a Philips EM 200 electron microscope. Toluidin-blue stain desections [27], 0·5–1 μm thick were used for

Table 1. *Evaluation of immunocytological (IFL and IEM) findings*

Cell preparations according to serum treatment	IFL	IEM				
	Pattern of membrane fluorescence	Total of cells examined	Cells with cytopathic changes[3]	Extracell. attached virus particles[4]	Fe label[5]	% cells labelled
A = Normal human + hybrid[1]	negative	80	2	3(10)	6[6]	0
B = Abwao + hybrid[1]	fine dotted and segmental	118	3	0(12)	10(2)	8
C = Abwao + anti Ig[2]	fine dotted, patchy and segmental type	92	6	1(9)	35(4)	38
D = Monkey-anti-Hela-cell + hybrid[1]	clearly stronger than B & C	83	2	2(10)	80(6)	96

[1]Hybrid treatment was followed by staining with apofluorretin (IFL) or ferritin (IEM) respectively.

[2]Antihuman Ig coupled with FITC (IFL) or ferritin (IEM) respectively.

[3]Changes characteristic of viral infection.

[4]The number in bracket indicates additional, equivocal, capsid-like particles.

[5]Ferritin label in groups B, C, and D means a few small clumps, patches, or a more extended row of ferritin attached, In brackets the number of cells is given which were heaviest labelled among the preceding number of cells, i.e. which had a segmental or nearly circumferential labelling.

[6]Single clumps of a few ferritin molecules, clearly distinct from specifically labelled areas in groups B, C, D.

selection of fields containing characteristically altered nuclei.

The immuno-electron microscopic findings are based on the analysis of approximately 400 micrographs and direct examination in the electron microscope of several hundred additional cells.

RESULTS

Membrane immuno-fluorescence (IFL)

The reactive cells showed a variable degree of membrane fluorescence after treatment with the Abwao serum, hybrid antibody and apofluorretin (Table 1B). A dotted to segmental type of membrane fluorescence was the usual pattern seen, (Fig. 1). The fluorescence was somewhat stronger with the conventional FITC conjugated anti-immunoglobulin as compared to the staining with hybrid antibody and apofluorretin, (Fig. 2), (Table 1C), but the fluorescent patterns were essentially the same. In preparations treated with heterologous anti-HeLa serum, (Table 1D) most cells showed a highly intensive ring type of fluorescence. Cells treated with normal human serum showed no membrane fluorescence, (Table 1A). A variable proportion of cells were damaged or dead as indicated by strong intracellular fluorescence.

Immuno-electron microscopy (IEM)

In parallel experiments, essentially the same qualitative results were obtained whether ferritin conjugated anti-Ig or hybrid antibody was used. However, there was a significantly larger number of cells labelled in cell preparations treated with ferritin labelled anti Ig, (Table 1C) as compared to cells treated with hybrid antibody and ferritin, (Table 1B). Also, the tagging with ferritin-conjugate was usually somewhat denser in terms of ferritin molecules per unit length (area) of membrane than the corresponding type of labelling with hybrid and ferritin. With respect to tagging with ferritin, the cells treated with the Abwao serum could be divided into three groups:

1. The majority of the cells (about 60%) showed no labelling in a given section plane. These cells generally appeared smaller than those in the other two groups. They had a smooth surface membrane or scanty microvilli, and no debris or virus particles attached to the surface. The nucleus and cytoplasm of these cells showed no conspicuous change.

2. The second group consisted of cells with one or a few patches with ferritin on their surface, no intracellular virus and no degenerative changes, (Fig 3). Virus particles similar to enveloped or non-enveloped EB virus, [20] were associated to the surface of some of these cells. The outer coat of these virus particles was consistently tagged with ferritin in cell preparations incubated with Abwao antiserum, (Table 1B, C), (Fig. 4, 5). In addition, labelled material, which morphologically could not be classified with certainty as virus particles was occasionally found associated with the surface of some of the cells, (Fig. 6) Usually no labelling of the cell membrane was observed at the sites of virus–membrane interaction. In several instances, virus particles appeared enclosed within membrane bound spaces near the cell border, (Fig. 7), presumably representing endocytic vacuoles or tangential cuts through deep indentations of the surface membrane. Cells of this group often had numerous surface villous projections. Vesicular material and debris was often loosely associated to the surface of these cells. The nucleus and cytoplasm of these cells were not altered.

3. A small number of cells (about 5%) showed a more extensive ferritin labelling of their surface membrane, (Fig. 8). Usually the tagging was present on relatively large areas or segments of the surface of the cells. All of these cells showed varying degrees of nuclear and cytoplasmic alterations indicating progressive degeneration and most of these cells contained EBV-type virus components in their cytoplasm and/or nuclei, [28], (Fig. 8, 9). Occasionally, extracellular virus particles were observed also near the surface of these cells.

Cells treated with the anti-Hela cell serum, (Table 1D) showed discontinuous segmental labelling of their surface membranes. A few virus particles were observed near the surface of some of these cells and occasionally showed labelling with ferritin particles. This ferritin tagging, however, was weaker than that observed on virus particles in cultures treated with Abwao serum, (Table 1B, C).

The cells treated with normal human serum showed labelling neither of their surface membrane nor of virus particles.

DISCUSSION

A previous report on the immuno-electron microscopy of MA and VCA activity of Burkitt cells using sera which were concordant and/or discordant for anti-MA or anti-VCA activity, indicated that MA was present on discrete membrane areas and on the envelope of extracellular virus particles and that anti-VCA sera labelled (extracellular) naked non-enveloped virus particles, [14]. The present study, using a concordant anti-MA and VCA

(Abwao) serum and a lymphoblastoid line (Ly-28) derived from a NPC, corroborates the results of Silvestre *et al.* with respect to the reactivity of extracellular virus and to the labelling of structurally nonspecialized membrane areas of the cells. The observed membrane labelling on Ly-28 cells most probably indicates MA distribution, since extracellular VCA is structurally linked to virus particles [14]. Two quantitatively different patterns of membrane labelling could be distinguished. The most prominent labelling was present on a relatively small percentage of cells, which were also recognized by cytopathogenic changes and evidence of characteristic viral structures, [20] in their nuclei and cytoplasm. These cells evidently release virus by disintegrating. This is consistent with the observations that most extracellular virus appeared to be of the non-enveloped type, and were labelled presumably due to the anti-VCA activity of Abwao serum. However, the distinction between enveloped and non-enveloped virus was not always possible due to dense ferritin labelling.

The more frequently observed other type of membrane reactive cells showed only small segments of the cell membrane labelled. Extracellular virus particles were often associated with the surface of these cells, but intracellular virus particles were not seen.

The presence of virus determined antigen on the surface of both types of cells suggests that they represent respectively early and late stages of virus infection [12, 17, 18]. Alternatively, the larger group of smaller cells with sparse surface labelling may only abortively express the MA antigen. Attachment of EBV particles to the surface of "nonproducer" cells has previously been observed [29]. The present study cannot exclude the possibility that some of the virus particles became attached to cells by crosslinking antibodies. However, this seems unlikely since in the present study, usually no labelling of the cell membrane was observed at the sites of virus-membrane interaction.

The findings of virus particles within deep cellular invaginations and membrane bound spaces could indicate that virus binding to the cell membrane can trigger an endocytic process.

In conclusion, the present observations seem to indicate the expression of EBV associated MA on discrete sites of the cell membrane of Ly-28 cells, without morphological evidence of virus, whereas cells containing virus showed a more extensive and continuous membrane labelling. Most virus particles attached to Ly-28 cells were of the non-enveloped type, presumably liberated from disintegrated cells.

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REFERENCES

1. M. A. Epstein and B. G. Achong, The EB Virus, in *Burkitt's Lymphoma.* (Edited by D. P. Burkitt and D. H. Wright) p. 231. Livingstone, London (1970).
2. G. Henle, W. Henle and G. Klein, Demonstration of two distinct components in the early antigen complex of Epstein–Barr virus-infected cells. *Int. J. Cancer* **8,** 272 (1971).
3. G. Klein, Herpesviruses and oncogenesis, *Proc. Nat. Acad. Sci.* **69,** 1056 (1972).
4. M. H. Miller, D. Stitt and G. Miller, Epstein–Barr antigen in single cell clones of two human leukocytic lines. *J. Virol.* **6,** 699 (1970).
5. K. Nilsson, G. Klein, W. Henle and G. Henle, The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. *Int. J. Cancer* **8,** 443 (1970).
6. G. de-Thé, Problèmes posés par les propriétés immunologiques communes au lymphome de Burkitt et au cancer du rhinopharynx. Moyens de les résoudre. *Ann. Inst. Pasteur* **122,** 767 (1972).
7. G. de-Thé, The etiology of nasopharyngeal carcinoma. In *Pathobiology Annual.* (Edited by H. Ioachim) p. 235. Appleton-Century-Crofts, New York (1972).
8. H. Zur Hausen, Epstein–Barr virus in human tumor cells. *Int. Rev. exp. Pathol.* **11,** 233 (1972).
9. G. Klein, P. Clifford, E. Klein and J. Stjernsvard, Search for tumor specific immune reactions in Burkitt lymphoma patients by the membrane immunofluorescence reaction. *Proc. nat. Acad. Sci.* **55,** 1628 (1966).

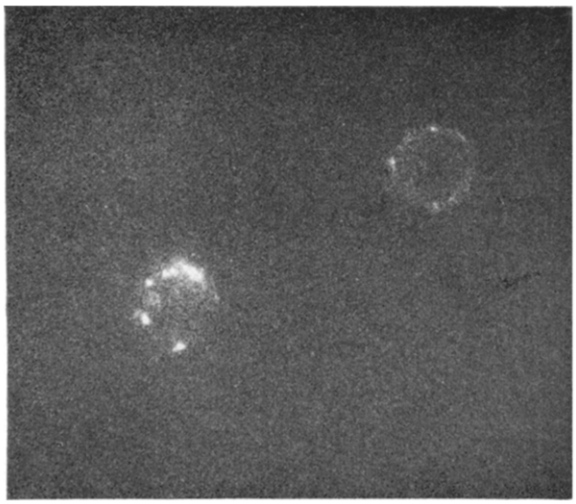


Fig. 1. *Fluorescence-micrograph of LY-28 cells treated with Abwao serum, hybrid and apofluorescein (cf. Table 1B). Note the varying degree of membrane fluorescence in a dotted and patchy pattern.* × 960.

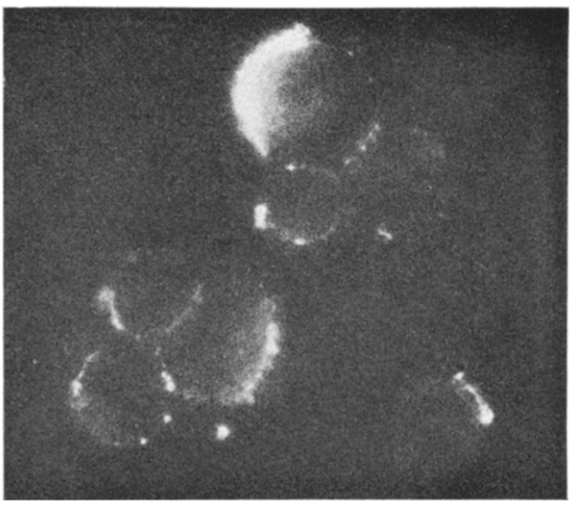


Fig. 2. *Membrane fluorescence of LY-28 cells treated with Abwao serum and Fitc anti-immunoglobulin. Note the somewhat more intensive staining as compared to Fig.* 1. *One cell upper center has a more continuous segmental or cap-like membrane reaction.* × 960.

(to face p. 636)

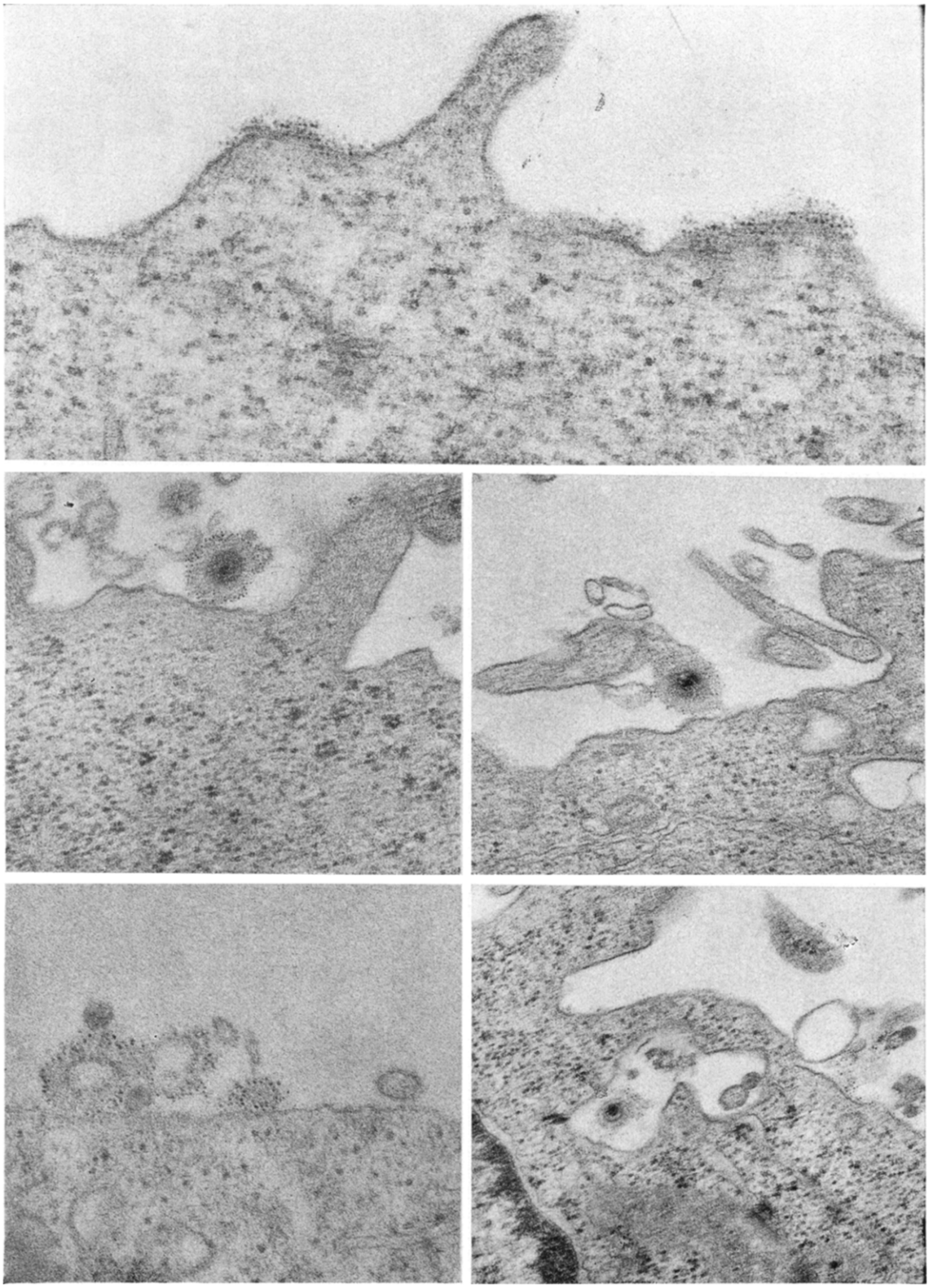


Fig. 3. *Electron micrograph showing patches of membrane (MA) labelling after treatment with Abwao serum and ferritin conjugated anti-immunoglobulin. (Table* 1, *C).* ×81,000.

Fig. 4. *Extracellular non-enveloped, coated virus in close association with the cell surface. Cell preparation treated with Abwao serum and ferritin conjugated anti-immunoglobulin (Table* 1, *C).* ×61,000.

Fig. 5. *Cell preparation treated with Abwao serum, hybrid and ferritin (Table* 1, *B). Note the somewhat less dense tagging of surface associated virus particle as compared to that of Fig.* 4. × 51,000.

Fig. 6. *Amorphous and vesicular material, presumably viral components occassionally found in association with the surface of cells.* ×81,000.

Fig. 7. *Labelled virus particle apparently enclosed within a vesicle formed by fusion of surface projections.* ×46,000.

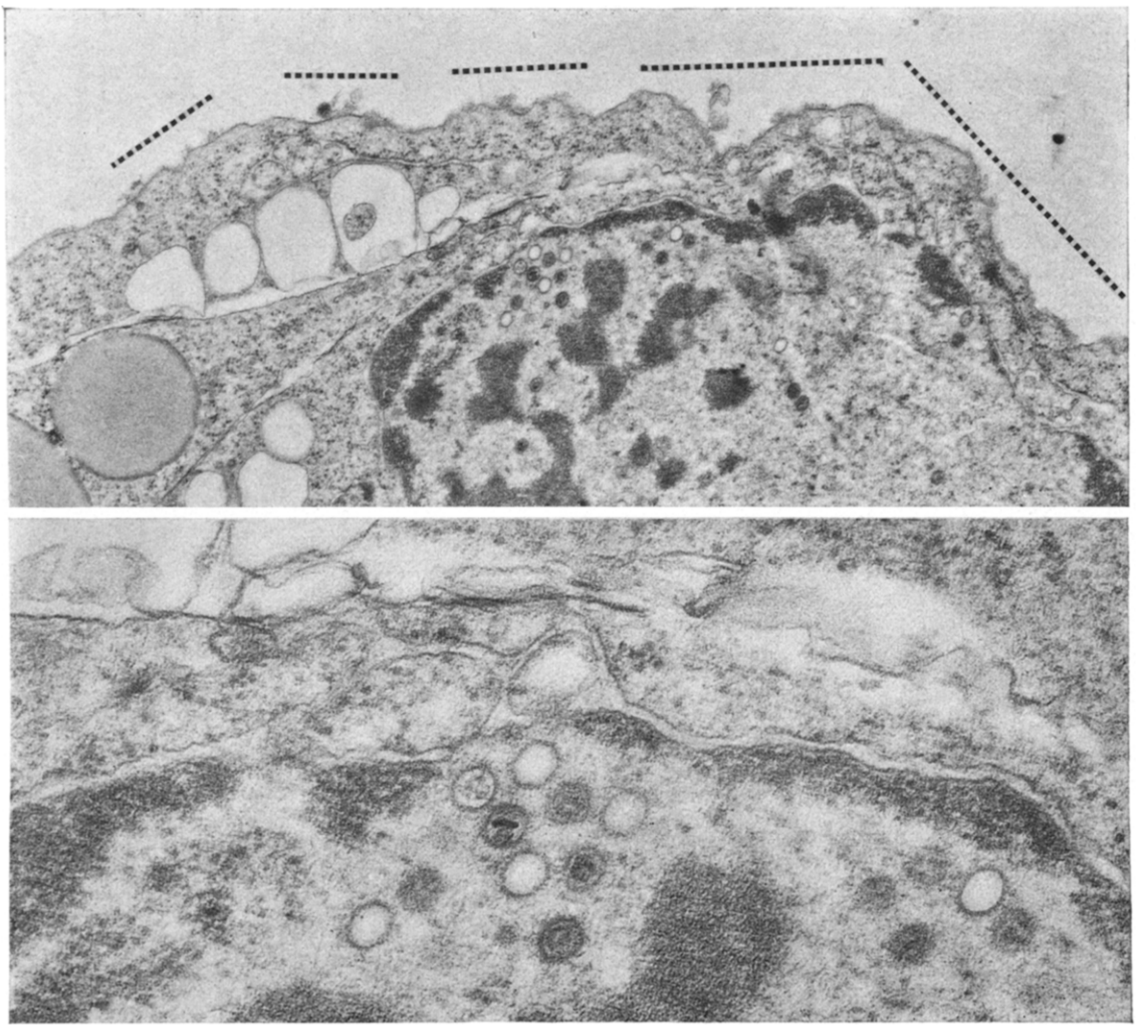


Fig. 8. *Virus containing large cell with characteristic cytoplasmic and nuclear alterations consistent with virus infection.* [1]. *Note the rather extensive labelling of the cell surface (delineated areas); cell preparation treated with Abwao serum and ferritin conjugate (Group C).* × 18,000.

Fig. 9. *Same cell as in Fig.* 8 *showing intranuclear viral components.* × 105,000.

10. 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).
11. A DE SCHRYVER, S. FRIBERG, G. KLEIN, W. HENLE, G. HENLE, G. DE-THÉ, P. CLIFFORD and H. C. Ho, Epstein–Barr virus (EBV)-associated antibody patterns in carcinoma of the postnasal space. *Clin. exp. Immunol.* **5,** 443 (1969).
12. G. KLEIN, P. CLIFFORD, E. KLEIN, R. T. SMITH, J. MINOWADA, F. M. KOURILSKY and J. H. BURCHENAL, Membrane immunofluorescence reactions of Burkitt lymphoma cells from biopsy specimens and tissue culture. *J. nat. Cancer Inst.* **39,** 1027 (1967).
13. G. KLEIN, E. KLEIN and P. CLIFFORD, Search for host defences in Burkitt lymphoma: Membrane immunofluorescence tests on biopsies and tissue culture lines. *Cancer Res.* **27,** 2510 (1967).
14. D. SILVESTRE, F. M. KOURILSKY, G. KLEIN, Y. YATA, G. NEAUPORT-SAUTES and J. P. LEVY, Relationship between the EBV membrane antigen on Burkitt lymphoma cells and the viral envelope, demonstrated by immunoferritin labelling. *Int. J. Cancer* **8,** 222 (1971).
15. G. KLEIN, G. PEARSON, J. S. NATKANY, J. J. NATKANY, E. KLEIN, G. HENLE, W. HENLE and P. CLIFFORD, Relation between Epstein-viral and cell membrane immunofluorescence of Burkitt tumor cells. I. Dependence of cell membrane immunofluorescence on presence of EB-virus. *J. exp. Med.* **128,** 1011 (1968).
16. G. KLEIN, G. PEARSON, G. HENLE, W. HENLE, V. DIEHL and J. C. NIEDERMAN, Relation between Epstein-viral and cell membrane immunofluorescence in Burkitt tumor cells. II. Comparison of cells and sera from patients with Burkitt's lymphoma and infectious mononucleosis. *J. exp. Med.* **128,** 1021 (1968).
17. W. HENLE, G. HENLE, B. A. ZAJAC, G. PEARSON, R. WAUBKE and M. SCRIBA, Differential reactivity of human serum with early antigens induced by Epstein–Barr virus. *Science* **169,** 188 (1970).
18. J. S. HOROSZIEWICZ, V. C. DUNKEL, L. AVILA and J. T. GRACE, EB virus infection and propagation in human hematopoietic cells. In *Comparative Leukemia Research,* Edited by R. M. DUTCHES, p. 722, S. Karger, Basel (1970).
19. L. GERGELY, G. KLEIN and I. ERNBERG, Appearance of Epstein–Barr virus associated antigens in infected Raji cells. *Virology* **45,** 10 (1971).
20. G. DE-THÉ, J. C. AMBROSIONI, H. C. Ho and H. C. KWAN, Lymphoblastoid transformation and presence of Herpes-type viral particles in a Chinese nasopharyngeal tumor cultured *in vitro. Nature (Lond.)* **221,** 770 (1969).
21. G. KLEIN and L. DOMBOS, Relationship between sensitivity of EBV-carrying lymphoblastoid lines to superinfection and the inducibility of the viral genome. *Int. J. Cancer* **11,** 327 (1973).
22. G. KLEIN, G. PEARSON, G. HENLE, W. HENLE, G. GOLDSTEIN and P. CLIFFORD, Relation between Epstein–Barr virus and cell membrane immunofluorescence and anti-EBV reactivities of different sera. *J. exp. Med.* **129,** 697 (1969).
23. P. GUNVEN, G. KLEIN, G. HENLE, W. HENLE and P. CLIFFORD, Antibodies to EBV associated membrane and viral capsid antigen in Burkitt lymphoma patients. *Nature (Lond.)* **228,** 1053 (1970).
24. P. BIBERFELD, G. BIBERFELD and P. PERLMANN, Surface immunoglobulin light chain determinants on normal and PHA-stimulated human blood lymphocytes studied by immunofluorescence and electron microscopy. *Exp. Cell Res.* **66,** 177 (1971).
25. G. GOLDSTEIN, Immunofluorescent detection of human antibodies reactive with tumor. *Ann. N.Y. Acad. Sci.* **177,** 279 (1971).
26. A. R. SPURR, A low viscosity epoxy-resin embedding medium for electron microscopy. *J. ultrastr. Res.* **26,** 31 (1969).
27. D. BENNETT and O. RADIMSKA, Flotation-fluid staining, toluidine blue applied to maraglas sections. *Stain Technol.* **41,** 349 (1966).
28. M. A. EPSTEIN, B. G. ACHONG and Y. M. BARR, Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* **i,** 702 (1964).
29. V. C. DUNKEL, J. S. HOROSZEWICZ and J. T. GRACE, Studies on the nature of surface immunofluorescence in EB infected cells. *Proc. Amer. Ass. Cancer Res.* **10,** 20 (1969).

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T-Lymphocyte Responses in Patients with Malignant Melanoma*

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Abstract—*Studies of lymphocyte transformation to phytohaemagglutinin (PHA), allogeneic lymphocytes (MLR) and purified protein derivative (PPD) were made in 71 patients with malignant melanoma.*

The MLR was clearly subnormal in 44%, due in half such patients to inhibitory factors in the serum and in the other half to intrinsically impaired lymphocytes.

Stage I, (10% absent MLR) were less affected than later stages.

Responses to PHA (97%) and to PPD (86%) were poor tests of a patient's ability to recognise foreign tissue antigens.

The MLR seems to be a much better test for assessing patients in relation to tumour-specific immunotherapy.

INTRODUCTION

MANY studies have been done on the immunological reactions which occur in patients with melanoma, both *in vivo* and *in vitro* [1–14]. These studies show that tumour specific antigens are present on the surface membrane and in the cytoplasm of malignant melanoma cells.

Humoral antimelanoma antibodies have been found in melanoma patients of Ugandan and Caucasian origin, by Lewis and co-workers. These antibodies were seen mostly in patients with early or localised tumours. Their presence, however, was not related to the total mass of the tumours present, and they did not prevent these patients from developing disseminated disease later.

In the last few years, there has been accumulating evidence that cancer patients have impaired cellular immunity, which may influence prognosis [15–17], and many methods have been used to study these cell mediated immune responses. Fass [4] showed that melanoma patients who retained delayed hypersensitivity responses to their autologous tumour extract had localised disease. In many other centres, the responses to other cutaneous test antigens, e.g. PPD, DNCB, streptokinase, streptodornase, Candida are under study. *In vivo*, immune defects have also been demonstrated by finding impaired skin allograft rejection [18], or disseminated disease following homologous immunisation with transplanted tumour cells [19]. *In vitro* responses have been studied by lymphocyte transformation to phytohaemagglutinin (PHA) [5], soluble tumour antigen isolated from melanoma cells [10], autologous tumour cells or extracts [7, 8], inhibition of leucocyte migration [6] and by lymphocyte-mediated cytotoxicity of melanoma cells [9].

Recently, serum inhibitory factor(s) which inhibit lymphocyte-mediated cytotoxicity, have been reported [11, 12]. The mixed lymphocyte reaction (MLR) is a monitor used *in vitro* to determine the recognition capabilities of one set of lymphocytes against the antigenicity of another set, blocked from blastogenesis by irradiation or mitomycin C. A response against these foreign antigens is characterised by blastogenesis of T-lymphocytes, measured by incorporation of radioactive thymidine. We could

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find no published studies of the MLR in malignant melanoma, or of serum inhibitory factors affecting the MLR of these patients and also those of healthy normal peoples' MLR. This communication reports such studies of T-lymphocyte responses.

MATERIAL AND METHODS

Patients

Seventy-one patients who had histologically proven malignant melanoma were studied, of which 30 were males and 41 were females. All these patients had PHA and MLR mediated transformation, performed in autologous and Blood Group ABO compatible sera of healthy donors. Of these patients, 42 had PPD performed simultaneously.

The Lewis *et al.*, [1] classification, with slight modification, was used in staging the diseases clinically:

Stage I	primary, with localised disease not affecting regional lymph nodes, and/or no distant metastases.
Stage IIa	local cutaneous recurrence. No regional lymph node metastases, and/or no distant metastases.
Stage IIb	local regional lymph node involved histologically. No distant metastases, characterised by normal chest X-ray, bone marrow, liver function test, blood urea and clinical examination.
Stage IIab	local cutaneous recurrence, with regional lymph node involved histologically, but no distant metastases (viz. IIb).
Stage III	distant metastases beyond anatomical region, proved histologically. All patients were blood group and HL-A typed.

Normals

These were normal healthy young adults, aged 20–45 years, (mean 28 years) who were blood group identical, but HL-A different. They were known to have normal T-lymphocyte cell function to PHA, MLR and PPD and their sera were known to contain no stimulatory or inhibitory activity. These individuals did not have previous blood transfusions, or pregnancies.

The melanoma patients investigated were aged between 21 and 73 years (mean 51 yr).

Preparation of lymphocytes

Lymphocyte transformation was performed according to the method of Yamamura, [20], blood specimens being collected preoperatively, and/or one month after chemotherapy, and/or radiotherapy. The lymphocytes were washed 3 times with medium TC199, counted and, without further purification, set up in triplicate cultures.

Control serum used in cultures

The stored serum of healthy ABO compatible but HL-A different individuals was used. Foetal calf serum was avoided because of its inhibitory effect on lymphocyte transformation. Similarly, non-identical ABO blood group serum was not used with the responding lymphocytes because it was found to stimulate or inhibit by about 20 % the lymphocyte transformation to PHA. Control subjects were healthy laboratory staff who had not previously been pregnant or had blood transfusions, and their sera were known to have no inhibitory or stimulatory activity.

Harvesting lymphocyte cultures

At the end of the appropriate incubation time, lymphocytes were harvested by centrifugation of the culture tubes. Harvested cells were washed with phosphate buffered saline and 5 % trichloracetic acid (TCA) added to lyse the red blood cells. The TCA precipitate was washed with methanol to remove precipitated haemoglobin, and the DNA precipitate was resuspended in scintillation fluid (NE 233, Nuclear Enterprises). The incorporated radioactivity was counted in a Tracerlab Liquid Scintillation Counter.

Transformation to PHA

One microgramme of purified phytohaemagglutinin (PHA, Burroughs Wellcome) was added to 2×10^5 lymphocytes with 10 % serum in a final volume of 1 ml. Normal control PHA responses were set up simultaneously with the patients' tests. Cultures were incubated for 48 hr and harvested as previously described. Results are expressed as Transformation Index (T.I.), which is the ratio of the average of triplicate stimulated cultures in counts/min over the counts/min of unstimulated cultures. The log-normal range (median ± 2SD) of T.I. is 27–93–255 [20].

Transformation to PPD

These cultures contained 10 μg purified protein derivative (PPD, Ministry of Agriculture Fisheries and Food, Weybridge). Each tube contained 2×10^5 lymphocytes and 20 % autologous serum in a culture volume of 1 ml. A similar set of lymphocyte cultures, from a

control, who was known to respond to PPD, was set up in triplicate as well as unstimulated cultures which contained lymphocytes, medium and saline. These cultures were incubated in 5% CO_2 at 37°C for 6 days. Tritiated thymidine was added for the last 16 hr of the incubation before harvesting (day 5–6). Results were expressed as T.I. and results above 2·5 were considered positive.

Transformation to allogeneic lymphocytes (MLR)

All MLR cultures were incubated for six days and harvested as described for PPD.

(a) *Patients' lymphocytes.* For the immunogen, allogeneic HL-A non-identical normal lymphocytes were incubated with mitomycin C (25 μg/ml, for 20 min at 37°C). Two-hundred thousand responding lymphocytes were mixed with an equal number of mitomycin C treated lymphocytes with 20% autologous patients' serum in a culture volume of 1 ml. The patients' lymphocytes were also washed 3 times and set up with control serum to see if there was any restoration of MLR.

(b) *Control lymphocytes.* The lymphocytes from healthy control were cultured in the presence of PHA, PPD and allogeneic normal mitomycin C treated lymphocytes (MLR) in autologous serum. MLR cultures were also set up in 10% and 20% of patients' serum to test for blocking activity.

(c) *Expression of results.* (i) A T.I. of below 2·5 was regarded as clearly subnormal. (ii) Improvement of the patient's MLR in ABO compatible serum was considered present when the T.I. rose above 2·5. (iii) Blocking of a normal's MLR occurred when the patient's serum reduced the T.I. by more than 50% when compared with that in autologous serum.

RESULTS

PHA responses

Nearly all patients, regardless of clinical staging of the disease, site of original primary lesion, age or sex, responded to PHA. Two patients with very advanced Stage III malignant melanoma had no response to PHA in autologous or in ABO compatible sera. Within a week of the results of their test, these 2 patients died from disseminated melanomatosis.

PPD responses

Forty-two patients had *in vitro* PPD responses studied at the same time as their PHA and MLR responses. Thirty-five patients (86%) had normal responses; while the 7 patients who did not have normal PPD responses had a normal MLR.

The PHA response was normal in all these patients, but there were 11 patients who had an absent MLR, and 9 of these had normal responses to PPD *in vitro* (Table 2). The presence of serum inhibitors did not appear to influence their PHA or PPD responses.

MLR responses

The T.I. of all patients and controls studied are shown in Fig. 1. In view of the poor

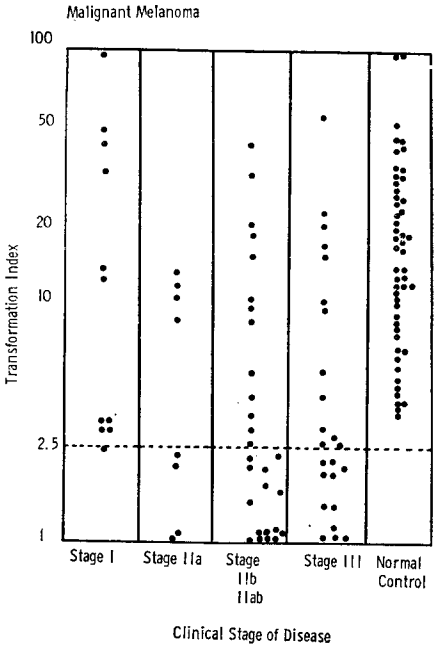


Fig. 1. MLR in autologous serum, using normal allogeneic HL-A non-identical, mitomycin-C treated lymphocytes as immunogens.

prognosis associated with stage of disease, site of primary lesion and sex of the patient, the result of the MLR study was analysed using these parameters.

(a) *Stage of disease.* (Table 1). The MLR was subnormal in 31 patients out of 71 cases of malignant melanoma studied, (44%). There were significantly more normal responders in Stage I when compared with other stages ($P < 0{\cdot}03$). There was a similar distribution of patients with subnormal MLR in Stage IIa, IIab, IIb and III ($P > 0{\cdot}1$).

(b) *Site of primary lesion.* The MLR results of patients with primary lesions of the upper limb, lower limb, trunk or head and neck were compared for immune competence in view of the better prognosis of limb lesions than that of trunk or head and neck lesions. Twenty-two of 44 patients with lower limb lesions (50%); 3 out of 8 patients with upper limb lesions (38%); 7

Table 1. *The relationship between the MLR and the stage of malignant melanoma*

	Total number of patients	Number of patients with absent MLR in autologous serum		
Stage I	11	1	(10%)	Stage I vs the rest $P < 0{\cdot}03$
Stage IIa	8	4	(50%)	Stage I vs Stage IIa and IIab $P < 0{\cdot}03$
Stage IIab	28	15	(53%)	Stage IIab vs III $P > 0{\cdot}1$
Stage III	24	11	(45%)	

out of 15 patients with trunkal lesions (50 %) and 1 out of 4 patients with lesions of the head and neck (25 %) showed subnormal MLR results. There was no significant difference in the distribution of a subnormal MLR between these sites and blocking activity in their sera was equally present.

(c) *Sex of patient* (see Table 3). There were 30 males and 41 females in the study. Fourteen of the 30 males and 17 of the 41 females had a subnormal MLR in autologous sera so that in overall frequency there was no sex difference ($P > 0{\cdot}1$). However, a difference in the nature of the subnormal MLR was present in that more males, 11 of 14, compared to 5 of 17 females had a subnormal MLR response in both autologous and ABO compatible sera indicating an intrinsic lymphocyte defect ($P < 0{\cdot}05$). Patients who had a subnormal MLR in autologous serum, but which was restored in ABO compatible normal serum were said to have serum inhibitors; these were present in 3 males (10 %) and 12 females (29 %) ($P < 0.1$).

Table 2. *The relationship between the absent MLR and the PPD responses. Examples are given for* 11 *patients who had normal PHA, but negative MLR in autologous serum*

Sex/Age	MLR in ABO serum	Blocking of normal MLR by 20% patients serum	PPD in autologous serum
F 54	+	+	+
M 66	+	+	0
F 43	+	+	0
F 29	+	0	+
M 48	+	0	+
F 68	0	+	+
M 68	0	+	+
M 47	0	0	+
F 56	0	0	+
M 26	0	0	+
M 49	0	0	+

Symbols + = normal response.
0 = absent response.

Table 3. *Nature of the absent MLR*

	Totals	Male	Female
Number of patients	71	30	41
Sub-normal MLR in autologous serum	31	14	17
Autologous serum inhibitors	15	3	12
Intrinsic lymphocyte defect	16	11	5*
Blocking of control MLR	16	5	11

*For this sex difference, $P < 0{\cdot}05$.

Blocking activity

The serum from 5 males (17 %) and 11 females (27 %) contained factors which inhibited the MLR of a normal control by greater than 50 % of the control's response in autologous control serum. Although more females showed inhibitory factors in their sera, this was not significant ($P > 0{\cdot}1$).

However, some patients had a subnormal MLR in autologous serum which was restored when tested in ABO compatible serum, but yet their sera did not necessarily block a control's MLR response, thus their inhibitory factors appeared to be specific only for the patient's own responses.

Conversely, 5 patients (3 females and 2 males) whose own MLR showed a T.I. greater than 2·5, had serum possessing inhibitory factors which affected the MLR of normal controls. Two of these females were in Stage I and IIa, respectively, and the others were all Stage III patients. The female patient in Stage I had persistent melanoma antibodies for the last three years with raised serum IgG, IgA and IgM.

In that no "normal range" existed for the MLR, it is possible that these 5 patients could have suppressed their own MLR but not below a T.I. of 2·5. In only 1 female (of all 71 patients) were multiple cytotoxic anti-HL-A antibodies

found. None of the males with serum inhibitors had received previous transfusions.

DISCUSSION

The importance of the absent mixed lymphocyte reaction

At the time the present study was commenced, attempts at a standardised MLR test were unsuccessful in that we were unable to store and recover a standard allogeneic lymphocyte mixture with a reproducible stimulus; fresh lymphocytes were always much better stimulators than those stored and revived. The rest of the test was optimised (as in methods), but the lack of a standard stimulus prevented the determination of a normal range for the MLR response. This may now have been overcome [21], and reports will appear soon from other workers. Notwithstanding, it was possible to recognise (Fig. 1) that all our normal subjects could register a transformation index of greater than 2·5. Therefore we have classified a response less than this as a subnormal MLR, and consider this as grossly abnormal as it has not been encountered in over 2000 MLR tests in over 300 normal subjects.

The most important finding of this study is the demonstration of a subnormal MLR in 31 out of 71 patients with melanoma, when lymphocyte transformation was done in the patients' own sera. Time course studies of the MLR in some of these patients using thymidine-uptake on days 3, 5, 7 and 9 did not reveal any variation from the normal maximum response at 5 days. Where repeated studies have been done in the individual patient, absent MLR has been a persistent finding. Nearly all these patients had normal responses to PHA and PPD. Of the 31 patients who did not have an MLR in autologous sera, 16 possessed inhibitory factor(s) in their sera, which depressed the MLR of normal young healthy adults (Table 3). In 11 of these patients, the MLR improved when their lymphocytes were washed and cultured in ABO-compatible sera. Depression of the MLR has been reported in patients with lung cancer [22], acute adult leukaemia [23], and with advanced non-lymphomatous cancers [21]. In leukaemia patients having immunotherapy with irradiated tumour cells and BCG, Powles [23] has found blocking activity in the sera of these patients, which inhibits the MLR of normal subjects. He avoided such inhibition by using foetal calf sera. We have avoided the use of foetal calf serum because in our laboratory, we find that it has a variable (often inhibitory) effect on lymphocyte transformation. Instead, we have used normal human ABO-compatible sera stored at −70°C. Our findings agree with the recent reports by Suciu-Foca and her colleagues [21], who found that the MLR of patients with advanced cancers (types unclassified) were impaired, that such MLR improved in pooled normal sera, and that the sera from such patients inhibited the MLR of normal healthy subjects.

We believe the MLR is currently the best available test to monitor the recognition capabilities of a subject for immune responsiveness to foreign tissue antigens. This would especially be so when potentially weak tumour transplantation antigens are under consideration. Our belief is based on: (i) logical extrapolation from MLR findings during many bone marrow transplantations, and other forms of homografts, (ii) inability of a few immune deficient patients to reject skin and other grafts when they had absent MLR yet PHA responsiveness within the normal range, (iii) failure, as yet, to show thymidine-uptake against tumour cells by lymphocytes taken after immunisation, where the preimmunisation tests have shown absent MLR, (iv) successful induction in 5 subjects of thymidine-uptake against tumour cells by post-immunisation lymphocytes where prior to immunization there had been a positive MLR.

The limited value of responsiveness to PHA or PPD

Transformation to PHA was only subnormal in 2 patients with advanced disease who both died within a week after the finding, so that the PHA test is a very poor assessment in the vast majority of melanoma patients, indeed it is not worth doing.

Similarly, responses to PPD were present in 86 % of these patients. Patients who had absent MLR could still have normal PHA and PPD responses; conversely absent PPD was found with positive MLR. The serum inhibitor(s) that affected their MLR and normal allogeneic lymphocytes did not appear to affect their own PHA and PPD responses. Thus PPD responsiveness is a bad test if the MLR really marks potential responsiveness to tumour antigens.

The aetiology of the absent MLR

Melanomata on the trunk, foot, hand, those that have ulcerated or have lymph node involvement have a poorer prognosis [24]. MLR responsiveness in melanoma patients appears to be significant only when Stage I patients, ($P < 0{\cdot}03$) are compared with other stages (Table 1). There were no significant

differences ($P > 0{\cdot}1$) in the incidence of inhibitory activity when related to site or sex distribution (Table 3).

Many of the women were in the post-menopausal age group. Only in one of these females were multiple antileucocyte antibodies found with cytotoxicity detected in HL-A systems. Fab fragments of equine antilymphocyte globulin, which are not cytotoxic to human lymphocytes, will inhibit the MLR [25]. It is possible that the MLR-blocking activity in these female patients may have been due to multiple *non-cytotoxic* anti-HL-A antibodies, developing as a result of previous sensitisation to pregnancy or blood transfusion. However, the difference in the incidence of blocking activity between males and females was not significant (Table 3). It is therefore likely that the blocking activity has arisen mainly in response to their melanomata. The presence of new iso-antigens has been detected in human neuroblastoma cells [26], prostatic carcinoma [27], and other tumours [28]. Suciu-Foca [21], in an extensive study of cancer patients who did not have previous transfusions found a relatively high incidence of anti-HL-A activity in cancer sera. Sjorgren *et al.* [29] have suggestive evidence that the "blocking antibodies" in tumour bearing individuals may be due to antigen–antibody complexes. Similarly, the lymphocyte cytotoxicity on melanoma cells has been found to be improved by washing 6 times [12].

Our own preliminary studies show that the lymphocytes of melanoma-bearing patients are poorly immunogenic in MLR studies to normal allogeneic adults. Such findings indicate that both the MLR responsiveness and the MLR stimulus can be deficient in lymphocytes from melanoma-bearing patients. This could be due to: (i) a firm coating (not readily washed off), or (ii) a loss of MLR expression on the lymphocyte surface, or (iii) a deletion of such cells from the peripheral blood of the patients, or (iv) a failure in other cell cooperation (e.g. phagocytes) needed for an MLR, or combinations of (i)–(iv).

The production of so-called "blocking antibodies" in transplantation and tumour systems, causing immunological enhancement has been suggested [30–32], and recent experiments have shown that there is a prolongation of rat kidney grafts, if antisera against donor kidney are given to the recipients [33]. Scanlon *et al.* [19] have described a fatal case of homotransplanted melanoma, when melanoma from the daughter was given to an 80-yr-old mother, who had suffered from adenocarcinoma of the endometrium 2 years earlier. Although no immunological studies on the MLR or of blocking activity were available, it is possible that one of the reasons why the graft took, was due to an absent MLR in the mother, or the presence of lymphocyte inhibitor(s) of her MLR by her previous carcinoma. Therefore, with the increasing interest shown in the use of tumour cell preparations, suitably inactivated, for active immunotherapy, it may be important to assess the recipient's MLR first, and then to monitor the MLR during immunisation to detect "blocking activity" and thereby the danger of immunological enhancement or of homografts taking. Perhaps part of the failure of various immunotherapeutic programmes, using tumour cell fractions, has been the administration of these cells, into tumour-bearing patients, with absent MLR and inhibitory factor(s) in their sera. The nature(s) of these "inhibitor(s)" of lymphocyte transformation of MLR is now under characterisation.

REFERENCES

1. M. G. Lewis, R. L. Ikonopisov, R. C. Nairn, T. M. Phillips, G. Hamilton Fairley, D. C. Bodenham and P. Alexander, Tumour-specific antibodies in human malignant melanoma and their relationship to the extent of the disease. *Brit. med. J.* **2,** 547 (1969).
2. M. G. Lewis, E. McCloy and J. Blake, The significance of humoral antibodies in the localization of human malignant melanoma. *Brit. J. Surg.* **60,** 443 (1973).
3. R. L. Ikonopisov, M. G. Lewis, I. D. Hunter-Craig, D. C. Bodenham, T. M. Phillips, C. I. Cooling, J. Proctor, G. Hamilton Fairley and P. Alexander, Autoimmunisation with irradiated tumour cells in human malignant melanoma. *Brit. med. J.* **2,** 752 (1970).
4. L. Fass, J. L. Ziegler, R. B. Herberman and J. W. M. Kiryabwire, Cutaneous hypersensitivity reactions to autologous extracts of malignant melanoma cells. *Lancet* **i,** 116 (1970).
5. W. J. Catalona, W. F. Sample and P. B. Chretien, Lymphocyte reactivity in cancer patients; correlation with tumour histology and clinical stage. *Cancer* **31,** 65 (1973).

6. A. J. COCHRAN, U. W. JEHN and B. P. GOTHOSKAR, Cell-mediated immunity in malignant melanoma. *Lancet* **i,** 1340 (1972).
7. G. A. NAGEL, W. F. PIESSENS, M. M. STILMANT and F. LEJEUNE, Evidence for tumour-specific immunity in human malignant melanoma. *Europ. J. Cancer* **7,** 41 (1971).
8. G. H. MAVLIGIT, U. AMBUS, J. U. GUTTERMAN, E. M. HERSH and C. M. MCBRIDE, Antigen solubilised from human solid tumours. Lymphocyte stimulation and cutaneous delayed hypersensitivity. *Nature (New Biol.)* **243,** 188 (1973).
9. G. A. CURRIE, F. LEJEUNE and G. HAMILTON FAIRLEY, Immunization with irradiated tumour cells and specific lymphocyte cytotoxicity in malignant melanoma. *Brit. med. J.* **2,** 305 (1971).
10. U. W. JEHN, L. NATHANSON, R. S. SCHWARTZ and M. SKINNER, *In vitro* lymphocyte stimulation by a soluble antigen from malignant melanoma. *New Engl. J. Med.* **283,** 329 (1970).
11. I. HELLSTROM, K. E. HELLSTROM, G. E. PIERCE and J. P. S. YANG, Cellular and humoral immunity to different types of human neoplasms. *Nature (Lond.)* **220,** 1352 (1968).
12. G. A. CURRIE, Effect of active immunization with irradiated tumour cells on specific serum inhibitors of cell-mediated immunity in patients with disseminated cancer. *Brit. J. Cancer* **28,** 25 (1973).
13. A. Z. BLUMING, C. L. VOGEL, J. L. ZIEGLER, N. MODY and G. KAMYA, Immunological effect of BCG in malignant melanoma. Two modes of administration compared. *Ann. intern. Med.* **76,** 405 (1972).
14. C. J. OON, P. ELLIOTT, H. BUCKLETON, J. WRIGHT, K. B. COOKE, M. APSEY and H. WHITSED, *In vitro* responses of human antimelanoma antibodies conjugated to chlorambucil on short term melanoma cell lines. *Third International Symposium on Malignant Melanoma*, Lyon. In press (1973).
15. F. R. EILBER, and D. L. MORTON, Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer* **25,** 362 (1970).
16. A. G. LEVIN, E. F. MCDONOUGH, JR., D. G. MILLER and C. M. SOUTHAM, Delayed hypersensitivity response to DNFB in sick and healthy persons. *Ann. N.Y. Acad. Sci.* **120,** 400 (1964).
17. A. C. SOLOWEY and F. T. RAPAPORT, Immunologic responses in cancer patients. *Surg. Gynaec. Obstet.* **121,** 756 (1965).
18. C. M. SOUTHAM, A. E. MOORE and C. P. RHOADS, Homotransplantation of human cell lines. *Science* **125,** 158 (1957).
19. E. F. SCANLON, R. A. HAWKINS, W. W. FOX and W. SCOTT SMITH, Fatal homotransplanted melanoma. *Cancer* **18,** 782 (1965).
20. M. YAMAMURA, Standardization of lymphocyte transformation to phytohaemagglutinin. *Clin. Exp. Immunol.* **14,** 457 (1973).
21. N. SUCIU-FOCA, J. BUDA, J. MCMANUS, T. THIEM and K. REEMTSMA, Impaired responsiveness of lymphocytes and serum-inhibitory factors in patients with cancer. *Cancer Res.* **33,** 2373 (1973).
22. T. HAN, and H. TAKITA, Impaired lymphocyte response to allogeneic cultured lymphoid cells in patients with lung cancer. *New Engl. J. Med.* **286,** 605 (1972).
23. R. POWLES and P. ALEXANDER, Possible occurrence *in vivo* of the autostimulating factor in lymphocytes. In *Birth Defects Original Article Series: Long Term Lymphocyte Cultures in Human Genetics.* Vol. 9, p. 111. National Foundation—March of Dimes, New York (1973).
24. Malignant Melanoma (Leader) *Brit. Med. J.* **2,** 129 (1967).
25. E. R. RICHIE, M. T. GALLAGHER, L. R. HEIM, M. A. SOUTH and J. J. TRENTIN, Inhibition of the mixed lymphocyte reaction with Fab fragments from anti-human ALG. *Proc. Soc. exp. Biol. (N.Y.)* **140,** 916 (1972).
26. J. BIEDLER, L. HELSON and N. SUCIU-FOCA, Expression of HL-A Antigens on Human Neuroblastoma cells in continuous culture. Abstracts of the 24th *Annual Meeting of the Tissue Culture Association,* Inc. (In press.)
27. R. J. ABLIN, Antibodies to lymphocytes in cancer. *Lancet* **i,** 41 (1972).
28. M. TAKASUGI and P. I. TERASAKI, Detection of HL-A and other cell-surface antigens on cultured cells by a cytotoxic plating inhibition test. *J. nat. Cancer Inst.* **49,** 1229 (1972).
29. H. O. SJOGREN, I. HELLSTROM, S. C. BANSAL and K. E. HELLSTROM, Suggestive evidence that the "blocking antibodies" of tumour-bearing individuals may be antigen–antibody complexes. *Proc. nat. Acad. Sci. (Wash.)* **68,** 1372 (1971).

30. N. KALISS, Immunological enhancement of tumour homografts in mice. *Cancer Res.* **18,** 992 (1958).
31. G. MOLLER and E. MOLLER, Immune cytotoxicity and immunological enhancement, In *Antibodies to Biological Active Molecules.* (Edited by B. CINANDER,) p. 349. Pergamon Press, Oxford (1966).
32. J. R. BATCHELOR, The use of enhancement in studying tumour antigens. *Cancer Res.* **28,** 1410 (1968).
33. J. R. BATCHELOR and V. C. JOYSEY, Influence of HL-A incompatibility on cadaveric renal transplantation. *Lancet* **i,** 790 (1969).

Europ. J. Cancer Vol. 10, pp. 647–651. Pergamon Press 1974. Printed in Great Britain.

Impulsecytophotometric Studies on the Effects of Daunomycin on Synchronised L-Cells*

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Abstract—*Mechanically synchronized cultures of L-cells were treated in various phases of the cell cycle with daunomycin. After incubation for 4 hr at a concentration of 1 μg/ml the percentage of cells in the various phases was determined using an impulse-cytophotometer†. Applying daunomycin in G_1 phase produced an arrest of cells in G_1 that had not been observed so far or had been considered negligible. When daunomycin was applied in S phase the progression of cells throughout this phase was not influenced and the end of S phase was not delayed, indicating that DNA synthesis of cells having already started DNA synthesis, is not disturbed by daunomycin at this low concentration. Daunomycin treatment in the late S phase or in the G_2 phase resulted in an accumulation of cells in G_2 (G_2 block).*

INTRODUCTION

ALTHOUGH daunomycin has been used successfully in the therapy of acute lymphatic and myelocytic leukemia [1, 2], its mechanisms of action are not yet fully understood. It has been postulated by various authors that daunomycin inhibits DNA synthesis more effectively than RNA synthesis [3, 4]. In proliferating mammalian cells daunomycin causes an arrest of cells in the G_2 phase of the cell cycle as has been demonstrated by Whang-Peng [5], Brehaut [6] and Göhde and Dittrich [7]. Studies of Kim *et al.* [8] on the lethal effects of daunomycin showed that the antibiotic is most toxic during the S phase and least during G_1 phase. In contrast, Silvestrini [9] found that the maximum inhibition of DNA synthesis is reached when the cells are treated in the middle of the preceding G_1 phase. In the present study the influence of daunomycin on the various phases of synchronized L-cell cultures is investigated by applying the method of impulsecytophotometry.

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†Impulsecytophotometry refers to the technique of flow microfluorometry and is relevant to the impulse-cytophotometer (ICP 11, Phywe, Göttingen).

MATERIAL AND METHODS

Cell culture

Mouse fibroblasts, line L-929, obtained from Dr. Mittermayer, Freiburg, were grown in monolayer culture in 1200 ml Roux flasks (Schott u. Gen., Mainz). The cell input per flask was 7×10^6 cells in 100 ml medium. After 24 hr confluent cultures were obtained. The culture medium was an Eagle's medium (No. 47340 A, Serva, Heidelberg) supplemented with 5 % fetal calf serum (Tissue Culture Services Ltd., Slough, Bucks.), penicillin and streptomycin. Subcultures were obtained by treatment with trypsin (0·05 %, Difco, 1:250).

Mechanical synchronization

The cells were synchronized in the absence of drugs by mechanical selection of mitotic cells [10]. For each experiment, we used 9 Roux flasks containing confluent cultures. By adding

20 ml of medium to one of the flasks and gently shaking mitotic cells were detached [10]. The medium was transferred subsequently into another flask and so forth. Nine bottles yielded about 6×10^5 cells. This procedure was repeated every 2 hr over a 32 hr period. The mitotic index increased with the number of synchronizations and reached a constant value of about 80 % after 6 hr. The first 3 sychronizations were discarded in all experiments. The synchronized cultures were transferred into 180 ml glass flasks (Schott u. Gen., Mainz) and incubated with medium at 37°C. Thus, 32 hr after the first synchronization, the synchronized cultures have reached an "age" of 0, 2, 4, etc. hr after mitosis covering the whole cell cycle of 23·6 hr. At this time, the control cultures were suspended in Tris buffer, fixed in methanol, and subsequently prepared for impulsecytophotometry. For mitotic index determination, cell suspensions of synchronized cultures were sedimented on microscopic slides by means of a sedimentation chamber [11]. The cells were fixed in absolute ethanol and stained with haemalaun. From each sample a total of at least 500 cells was scored for mitotic figures.

Daunomycin treatment*

Immediately after the last synchronization (i.e. 32 hr after the first shaking), the synchronized cultures were treated with daunomycin at a concentration of 1 μg/ml. Thus, the daunomycin treatment was done at different cell ages (0, 2, 4 etc. hr) after mitosis. After 4 hr of incubation at 37°C, the cells were fixed in methanol and prepared for impulsecytophotometry.

Impulsecytophotometry

The impulsecytophotometer (ICP) has been described by Dittrich and Göhde [12, 13]. It allows a quick determination of the DNA content of single cells in suspension after staining with a fluorescent dye, thus providing DNA distribution patterns (DNA histograms) of cell populations. Cells for investigation with the ICP were prepared as described previously [14]. The methanol fixed cells were washed, treated with RNAase (1 hr) and pepsin (15 min) and stained with the fluorescent dye, ethidium bromide. Under these conditions, the amount of dye bound is proportional to the DNA content of the cells [13]. Unbound ethidium bromide, as well as daunomycin incorporated in the cell, gives no measurable contribution to the fluorescence of the ethidium bromide DNA complex. Each DNA distribution represents ICP measurements from 40,000 cells, taking 1–3 min to complete depending on cell concentration. The DNA distribution was plotted on a chart recorder and digitally transferred onto punched tape. The quantitative analysis of the data was performed by a computer program giving as a result the percentage of G_1, S and $G_2 + M$ cells [15].

*Daunoblastin®, Farmitalia, Freiburg, F.R.G.

RESULTS

The progression of mechanically synchronized L-cells through the cell cycle is shown in Fig. 1. The DNA distribution patterns represent cells at different ages after synchronization in mitosis. The diagrams are selected from a series

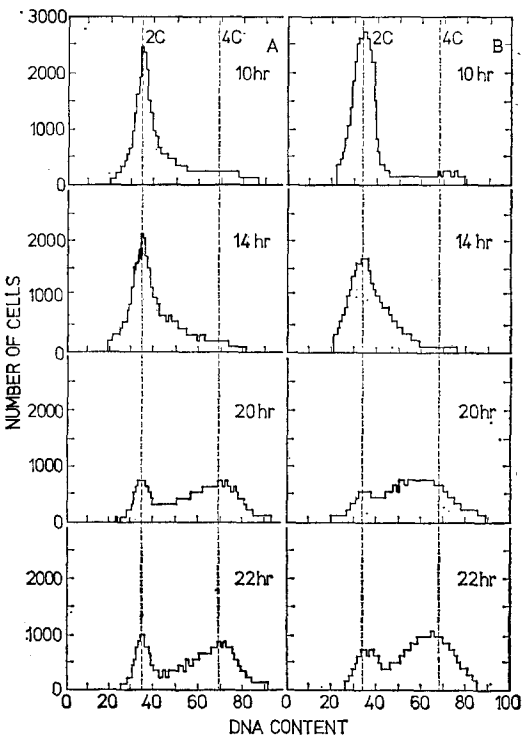


Fig. 1. DNA histograms of partially synchronized L-cell cultures at different times after synchronization (mitosis) obtained with the impulsecytophotometer. Total number of cells: 40,000 per sample. (a) untreated controls. (b) treated with 1 μg/ml daunomycin for 4 hr just before measurement.

taken at 2 hr intervals. The left peak represents cells in G_1 (DNA content 2 C), the right peak, cells in $G_2 + M$ with double the DNA content (4 C). The area between the 2 peaks corresponds to S-cells with variable DNA content between 2 C and 4 C. At a cell age of 10 hr, the cells of the control cultures begin to enter the S phase (Fig. 1A). It can be seen from the asymmetry of the G_1 peak in the 14 hr histogram that the number of cells in S phase has in-

creased. At about 20 hr after synchronization, the cells of the untreated controls are leaving the S phase, and at 22 hr the maximum of cells has reached G_2 phase. The corresponding histograms for the daunomycin treated cells are presented in Fig. 1B. We see that cells treated in G_1 at 6 hr (10 hr histogram) are blocked from entering S phase, whereas cells treated in early S phase at 10 hr (14 hr histogram) progress normally. At a time of 20 hr no differences between daunomycin treated cultures and controls can be seen. Twenty-two hours after synchronization, the treated cells are accumulated in the G_2+M phase.

A mathematical confirmation of the daunomycin induced arrest of cells in G_1 was obtained by calculating the percentage of cells in the different phases of the cell cycle at the time of G_1/S transition (10 hr after synchronization). At this time in the control cultures, $56 \pm 3\%$ of cells are in G_1 whereas in the daunomycin treated cultures $78 \pm 2\%$ of cells are in this phase (mean values $\pm$ S.E. from 6 repetitive experiments). This observation is supported by the reduced percentage of treated cells in S phase ($8 \pm 2\%$ compared with $31 \pm 3\%$ in the controls). A statistical calculation using the *t*-test proves the differences to be significant at $P < 0{\cdot}001$.

The duration of the phases in the cell cycle can be estimated from Fig. 2; giving the percentage of cells in the different phases over a total time of 26 hr after synchronization. Taking the time when the S curve reaches half of its maximum value as the beginning of S phase, the duration of G_1 is 10 hr for the control cultures (Fig. 2A). Using the same criterion (half the maximum of the G_2+M curve), the G_2 phase begins at a cell age of 20 hr, giving a duration of 10 hr for the S phase. Daunomycin treatment in the G_1 phase causes, under our experimental conditions, a delay in the beginning of S phase of at least 4 hr (Fig. 2B). The further progression of cells through the S phase after daunomycin treatment in G_1 phase was not investigated. Daunomycin application in S phase did not affect the end of S phase since the half-maximum value of the G_2+M fraction occurs at 20 hr in both control and treated cultures.

The accumulation of cells in G_2+M, at a cell age of 22–24 hr, can be seen from the differences of the G_2+M curves. The maximum percentage of cells in G_2+M is about 60 % in the treated cultures (Fig. 2B) compared to 45 % in the controls (Fig. 2A). As G_2 and mitotic cells are represented as a single peak in the DNA distribution, we determined the mitotic index by the evaluation of microscopic slides. Synchronized cultures treated with daunomycin during mitosis showed less than 1 % mitotic figures 4 hr later, proving that

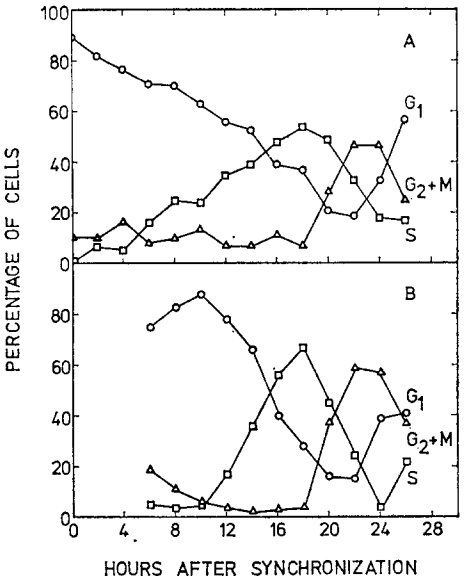


Fig. 2. Percentage of cells in the different phases of the cell cycle for partially synchronized L-cell cultures. (a) Untreated controls. (b) Treated with 1 μg/ml daunomycin for 4 hr just before measurement. G_1 (○), S (□), G_2+M (△). The results were obtained by mathematical analysis of the respective DNA histograms.

daunomycin causes no mitotic arrest. Thus, the observed accumulation of cells in G_2+M is an arrest in the G_2 phase.

DISCUSSION

Our results demonstrate that daunomycin treatment in the G_1 phase causes a pronounced arrest of synchronized L-cells in this phase. This finding is in contrast to the results of Tobey [16] for Chinese hamster cells showing that daunomycin at this dosage level had only a slight effect upon the entry of cells into S phase. The arrest of cells in the G_1 phase results in a delay of the S phase. It has not been tested whether the cell progression through the S phase is affected by daunomycin treatment in G_1 phase. The arrest of cells in G_1 by treatment with daunomycin might be explained by a block at the G_1/S transition. On the other hand, daunomycin could also slow down the traverse of cells through the entire G_1 phase. Our data do not settle this question. Wilms and Wilmanns [17] found an increased activity of the enzymes, DNA polymerase and thymi-

dine kinase in human leukocytes after daunomycin treatment. This might support the suggestion of a G_1/S block. The arrest of cells at the G_1/S transition could either induce the production of these enzymes or just increase the quantity of enzyme produced by the greater number of cells in this stage of the cell cycle. On the other hand, the biochemical findings cited could also be caused by alterations in the S phase.

Another important finding was that the application of 1 μg/ml daunomycin in S phase did not influence cell progression through the S phase at all. The S phase was completed at a cell age of 20 hr as it was in the controls. We therefore suggest that at least for this low concentration of daunomycin, cells that have started DNA synthesis are not affected. The treated cells synthesize a full complement of DNA at the same rate as the controls. Silvestrini [9] also found that maximum inhibition of DNA synthesis in synchronized cells (rat fibroblasts) by daunomycin was not achieved by treatment in S phase but in the middle of G_1 phase. We suggest that the inhibition of DNA synthesis as reported by several authors [3, 4, 8, 18, 19] may be caused at least partially by the arrest of cells at the G_1/S transition.

In a previous investigation with an asynchronous L-cell culture we demonstrated the accumulation of cells in G_2 after treatment with daunomycin [14]. Similar findings have been reported by other authors. Whang Peng *et al.* [5] and Brehaut [6] found an arrest in G_2 for daunomycin treated human leukocytes *in vitro*. Also, the impulsecytophotometric studies of Göhde and Dittrich [7], on the effects of daunomycin on the Ehrlich Ascites Carcinoma *in vivo* showed the reversible accumulation of cells in the G_2 phase. The G_2 block might be explained by the inhibition of ribosomal RNA synthesis as found by Dano *et al.*, [19]. Silvestrini [9], however, assumes that the inhibition of additional metabolic processes plays an important role.

Daunomycin has been assumed to be a possible synchronizing agent for human tumour cells *in vivo*. As our results have shown that daunomycin inhibits cell progression both in the G_1 and the G_2 phase, the successful application of this drug for the purpose of tumour cell synchronization must remain in doubt.

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REFERENCES

1. P. Obrecht, H. Merker, H. H. Hennekeuser, G. Meuret, M. Westerhausen and A. Simon, Klinisch-pharmakologische, cytochemische und Feulgen-photometrische Untersuchungen bei unreifzelligen Erwachsenenleukosen unter kombinierter Chemotherapie mit Vincristin, Daunorubicin and Prednison. *Klin. Wschr.* **48,** 1281 (1970).
2. M. Weil, O. J. Glidewell, C. Jacquillat, R. Levy, A. A. Serpick, P. H. Wiernik, J. Cuttner, B. Hoogstraaten, L. Wasserman, R. R. Ellison, S. Gailam, K. Brunner, R. T. Silver, V. B. Rege, M. R. Cooper, L. Lowenstein, N. I. Nissen, F. Haurani, J. Blom, M. Boiron, J. Bernard and J. F. Holland, Daunorubicin in the therapy of acute granulocytic leukemia. *Cancer Res.* **33,** 921 (1973).
3. V. Bremerskov and R. Linnemann, Some effects of daunomycin on the nucleic acid synthesis in synchronized L-cells. *Europ. J. Cancer* **5,** 317 (1969).
4. A. Theologides, J. W. Yarbro and B. J. Kennedy, Daunomycin inhibition of DNA and RNA synthesis. *Cancer* **21,** 16 (1968).
5. J. Whang-Peng, B. G. Leventhal, J. W. Adamson and S. Perry, The effect of daunomycin on human cells *in vivo* and *in vitro*. *Cancer* **23,** 113 (1969).
6. L. A. Brehaut, A delay in the G_2 period of cultured human leukocytes after treatment with rubidomycin (daunomycin). *Cell Tiss. Kinet.* **2,** 311 (1969).
7. W. Göhde and W. Dittrich, Die cytostatische Wirkung von Daunomycin im Impulscytophotometrie Test, *Arzneimittel-Forsch.* **21,** 1656 (1971).
8. J. H. Kim, A. S. Gelbard, B. Djordjevic, S. H. Kim and A. G. Perez, Action of daunomycin on the nucleic acid metabolism and viability of HeLa cells. *Cancer Res.* **28,** 2437 (1968).
9. R. Silvestrini, A. Di Marco and T. Dasdia, Interference of daunomycin with metabolic events of the cell cycle in synchronized cultures of rat fibroblasts. *Cancer Res.* **30,** 966 (1970).

10. C. MITTERMAYER, P. KADEN and W. SANDRITTER, Untersuchungen an teilungssynchronen L-Zellen. I. Optimale Wachstumsbedingungen und Charakterisierung des synchronen Zellsystems. *Histochemie* **12,** 67 (1968).
11. J. L. J. GAILLARD and A. SCHABERG, A new spreading procedure for human chromosomes. *Exp. Cell Res.* **36,** 415 (1964).
12. W. GÖHDE and W. DITTRICH, Impulsfluorometrie—ein neuartiges Durchfluß̈verfahren zur ultraschnellen Mengenbestimmung von Zellinhaltsstoffen. *Acta histochem. (Jena), Suppl.* **10,** 429 (1971).
13. W. GÖHDE, W. DITTRICH, K. H. ZINSER and J. PRIESHOF, Impulszytophotometrische Messungen an atypischen Zellabstrichen aus Scheide und Cervix uteri. *Geburtsh. u. Frauenheilk.* **32,** 382 (1972).
14. W. A. LINDEN, F. ZYWIETZ, H. LANDEN and C. WENDT, Synchronisation des Teilungszyklus von L-Zellen in der G_2-Phase durch fraktionierte Röntgenbestrahlung und Daunomycin. *Strahlentherapie* **146,** 216 (1973).
15. H. BAISCH, W. GÖHDE and W. A. LINDEN, Cell cycle analysis by impulsecytophotometry. *Rad. Environ. Biophys.* (In press).
16. R. A. TOBEY, Effects of cytosine arabinoside, daunomycin, mithramycin, azacytidine, adriamycin, and camptothecin on mammalian cell cycle traverse. *Cancer Res.* **32,** 2720 (1972).
17. K. WILMS and W. WILMANNS, Die Wirkung von Dauno-Rubidomycin und Adriamycin auf Enzyme der DNS-Synthese in Leukocyten *in vivo* und in der Kultur. *Klin. Wschr.* **50,** 866 (1972).
18. W. D. MERIWETHER and N. R. BACHUR, Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. *Cancer Res.* **32,** 1137 (1972).
19. K. DANØ, S. FREDERIKSEN and P. HELLUNG-LARSEN, Inhibition of DNA and RNA synthesis by daunorubicin in sensitive and resistant Ehrlich ascites tumor cells *in vitro*. *Cancer Res.* **32,** 1307 (1972).

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In Vitro Binding of Radioiodinated Human Placental Lactogen to Murine Mammary Gland

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Abstract—In vitro *binding of radioiodinated human placental lactogen (^{125}I-HPL) to homogenates of the mouse mammary gland was studied. The hormone binding components were susceptible to proteolytic enzymes. Differential centrifugation studies revealed the existence of hormone binding components in fractions of mammary gland homogenates sedimentable at* 800 ***g***, 10,000 ***g*** *and* 100,000 ***g***. *The binding was affected by incubation time and hormone concentration. Theophylline increased the binding of ^{125}I-HPL to mammary gland. Comparative studies on binding capacity of mammary gland of mice resistant and susceptible to spontaneous development of breast cancer suggest that mammary glands of C3H (Jax) mice have significantly higher binding capacities than those of C57 BL.*

INTRODUCTION

THE role of prolactin in mammary gland growth, development and carcinogenesis is investigated. It has been recently demonstrated that there exists a prolactin-like substance (placental lactogen) in the placentae of mice [1], rats [2–8], goats [9, 10] and monkeys [11–14] as well as humans [8, 15]. The similarity of placental lactogen to prolactin in several biological actions has been documented [16, 17]. *In vitro* studies carried out by Turkington and Topper [18, 19] have indicated that human placental lactogen (HPL) and prolactin are equally effective in inducing alveolar development and casein synthesis in mammary tissue of pregnant and non-pregnant mice. These authors have further demonstrated the similarities between the actions of HPL and prolactin on mouse mammary gland. The possible role of placental lactogen in certain mammary tumors that grow rapidly in mice during pregnancy and regress after parturition has been discussed by Fould [20]. Recently Yanai and Nagasawa [21] have reported that HPL injected into ovariectomized C3H/He mice plays an important role in maintenance and growth of hyperplastic nodules. Such interesting findings on the effects of this hormone led us to study the mechanism of its action on mammary tissue. The earliest event in the mechanism of action of protein hormones seems to be their binding to receptors in or on the cells of the target tissue. A number of recent studies has demonstrated the presence of specific binding components for peptide hormones in their respective target tissues [22–28]. The present studies were carried out to demonstrate and characterize the interaction between human placental lactogen and the components of mouse mammary gland tissue, in an *in vitro* system.

Since we have been interested also in mouse mammary carcinogenesis [29–33], studies of the binding capacity of the mammary gland of susceptible and resistant strains of mice were included as well.

Accepted 20 May 1974.

MATERIAL AND METHODS

Biological

Female mice of suceptible C3H (Jax) and resistant C57 Bl strains were obtained from our animal colony at the Cancer Research Institute, Bombay.

Chemical

Human placental lactogen (HPL), Human chorionic gonadotropin (HCG), Human luteinizing hormone (HLH), and Human follicle stimulating hormone (HFSH) used for the studies were gifts from NIAMD of the National Institute of Health, Bethesda, U.S.A. Trypsin, chymotrypsin and collagenase were obtained from Worthington Biochemical Corporation, U.S.A., and proteinase, ribonuclease, deoxyribonuclease were obtained from Koch-light Laboratories, U.K. Toluene sulphonyl arginine methyl ester (TAME) was supplied by Mann Research Laboratories Inc., U.S.A. Carrier free ^{125}I was obtained from the Radiochemical Centre, Amersham, England.

The method of Greenwood *et al.* [34] as modified by Midgley [35] was used to iodinate HPL, HFSH, HLH and HCG. The specific activities of the labelled hormones ranged from 100 to 150 μCi/μg. To discover the extent of hormone damaged during iodination, ^{125}I-labelled HPL was precipitated by antibody to HPL. It was found that 90–95 % of the labelled hormone could be precipitated by the antibody. These results indicated that damage due to iodination was very little.

Four-month-old virgin female mice of C3H (Jax) strain were used throughout the experiment except in one experiment where virgin female C57 BL mice were also used. Mice were killed by cervical dislocation and breast tissue was dissected out immediately, minced finely with scissors and homogenised in a Potter–Elvehjem homogenizer, in cold 0·01 M phosphate buffer (pH 7·0) containing 0·14 M sodium chloride (PBS) and made up to 15 % tissue concentration with cold PBS. The homogenate was centrifuged at 800 *g* in cold at +4°C so as to collect the fat layer. After its removal, the supernatant and precipitate were mixed thoroughly and used as such. In all experiments, tissues were pooled from 3 to 4 animals and results included in the paper are based on observations made on 3–4 such pools of tissues.

Binding procedure

The following method of binding was employed in our assay system unless otherwise specified. Aliquots of tissue homogenate were incubated with ^{125}I-HPL in a final volume of 0·5 ml made up with PBS, at 37°C for 60 min in a Dubnoff metabolic shaker (80 strokes per min). At the end of the incubation period, the contents of each tube were diluted to 2·5 ml with PBS containing 0·1 % gelatin (PBS-Gel) and centrifuged at 800 *g* for 10 min at 4°C. The supernatant was decanted off and the pellet washed twice with 3 ml of PBS-Gel. The tubes were drained and the amount of radioactive hormone bound to the pellet was determined by gamma ray spectrometry. Tubes containing PBS-Gel, served as controls for determining non-specific adherence of radioactive material to glass. All the determinations were carried out at least in duplicate. The results were expressed as a percentage of total added counts which were bound to the pellet. In all the experiments spleen was used as a control tissue to find out the extent of hormone non-specifically bound (adsorbed hormone). It was observed that binding of ^{125}I-labelled HPL to 800, 10,000 and 100,000 *g* pellets of spleen was respectively 1, 2 and 2·4 %. Protein content was measured by the method of Lowry *et al.* [36], using bovine serum albumin as standard.

(1) *Effect of tissue concentration.* Aliquots of homogenate representing 3 mg to 12 mg of proteins in the whole homogenate were incubated with 3·0 ng of ^{125}I-HPL in a final volume of 0·5 ml at 37°C for 60 min.

(2) *Effect of hormone concentration.* Nought point four millilitre aliquots of homogenate of mammary gland were incubated with increasing amounts of ^{125}I-HPL ranging from 3·0 ng to 24·0 ng at 37°C for 60 min, in a final volume of 0·5 ml.

(3) *Effect of time.* Nought point four millilitre aliquots of homogenate of mammary gland were incubated at 37°C for intervals of time ranging from 30 to 180 min, with 3·0 ng of ^{125}I-HPL in a final volume of 0·5 ml. A parallel series of tubes, containing an additional 1·0 mg of TAME per tube were run.

(4) *Effect of unlabelled HPL on binding of ^{125}I-HPL to 800 g pellet of mammary gland.* Nought point four millilitre aliquots of homogenate of mammary gland were incubated with 3·0 ng of ^{125}I-HPL in the presence of 25 ng to 200 ng of unlabelled hormone at 37°C for a period of 60 min.

(5) *Subcellular localization of binding components of the mammary gland.* One millilitre aliquots of homogenate were incubated with 6·0 ng of ^{125}I-HPL in a final volume of 1·5 ml at 37°C for 60 min. After incubation, the tubes were centrifuged to collect the 800 *g* pellet. The super-

natant was diluted to 12·0 ml with 0·25 M sucrose and centrifuged in a Spinco model L-65 using a fixed angle rotor to obtain 10,000 (mitochondrial), and 100,000 *g* (microsomal) fractions.

(6) *Effect of enzyme digestion.* To ascertain the nature of binding material present in 800 and 10,000 *g* fractions from mammary gland homogenates, the pellets were pre-treated with various degradative enzymes. Eight-hundred and 10,000 *g* pellets of mammary gland homogenate were prepared and resuspended in PBS to the original volume of the homogenate. Nought point four millilitre aliquots of the suspension were incubated at 37°C for 30 min with 0·25 ml of different enzyme solutions containing 250 μg of the enzyme. The tubes were centrifuged, the pellets were washed twice with PBS-Gel and resuspended in 0·4 ml of PBS-Gel, 3·0 ng of ^{125}I-HPL were added and the tubes incubated at 37°C for 60 min.

(7) *Effect of theophylline on binding.* Aliquots of tissue homogenate were incubated with 0·4, 1·2 and 1·6 mg of theophylline and ^{125}I-HPL in a final volume of 0·6 ml at 37°C for 60 min.

(8) *Binding of* ^{125}*I-labelled HPL, HCG, HLH and HFSH to mammary gland.* Nought point four millilitre aliquots of homogenate of mammary tissue were incubated with any one of the above mentioned labelled hormones, at 37°C for 60 min.

(9) *HPL binding to mammary gland of virgin female mice of C3H (Jax) and C57 BL strains.* Mammary glands of individual mice were homogenized in PBS. Nought point four millilitres of homogenate was incubated with 3·0 ng of ^{125}I-HPL at 37°C for 60 min. Four mice from each strain were used for each experiment. Results are expressed as a percentage of initial total hormone bound per mg of protein in the homogenate.

RESULTS

Figure 1 illustrates the effect of tissue concentrations on binding. It may be noted that the percentage of ^{125}I-HPL bound to 800 *g* pellet increases linearly with the various concentrations of the homogenate used.

It may be noted from Fig. 2 that there is a linear relationship between the percentage of hormone bound to 800 *g* pellet and the concentration of ^{125}I-HPL added to tissue homogenate in the incubation mixture. The range of hormone added was from 3 to 25 ng and the corresponding binding ranged between 30–240 pg.

Subsequent experiments were carried out to study the effect of the lengths of incubation periods with and without TAME (Fig. 3). The maximum binding without TAME occurred at 120 min, decreasing at 180, and 240 min. In the presence of proteolytic enzyme inhibitor TAME, the binding capacity of the 800 *g* pellet was increased, as compared with the corresponding binding without TAME, at all time intervals

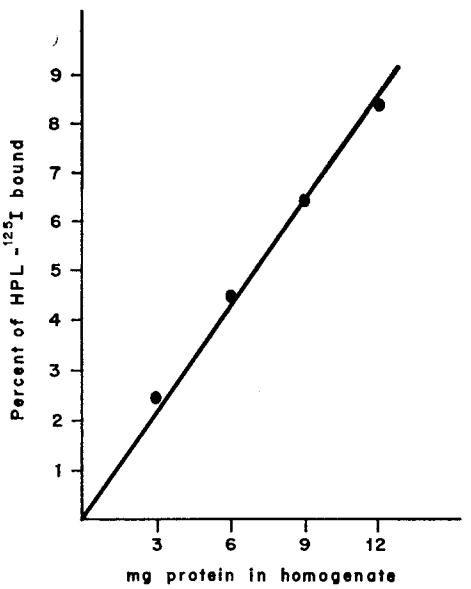


Fig. 1. *Effect of homogenate concentration on binding of* ^{125}*I-HPL to* 800 *g pellet of mouse mammary gland. Each point represents mean of duplicate observations.*

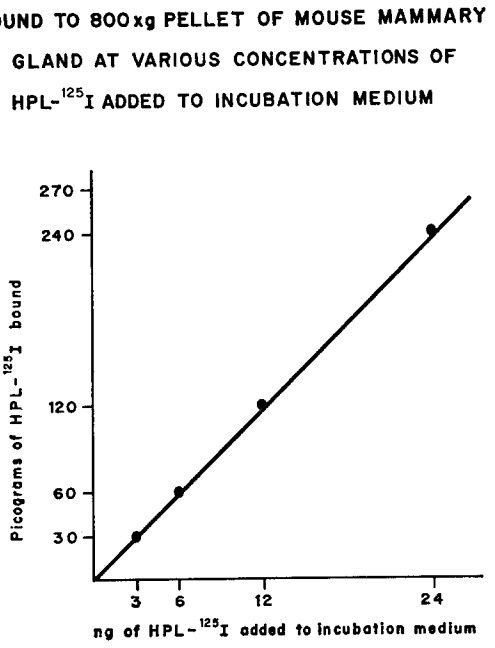


Fig. 2. *Picograms of* ^{125}*I-HPL bound to* 800 *g pellet of mouse mammary gland at various concentrations of* ^{125}*I-HPL added to incubated medium. Each point represents mean of duplicate observations.*

studied. There is a steady increase in binding from 30 to 180 min in presence of TAME, as compared to non-detectable binding at 30 min and increase in binding up to 120 min in absence of TAME.

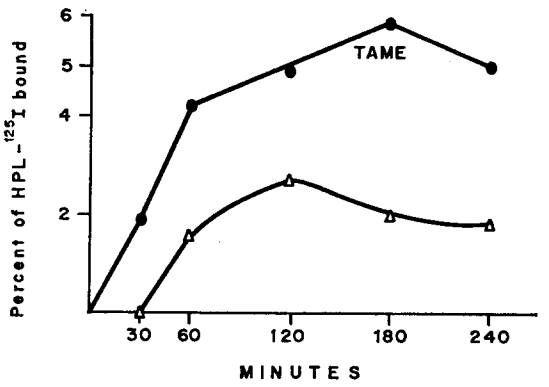


Fig. 3. Effects of time and TAME (Toluene sulphonyl arginine methyl ester) on binding of ^{125}I-HPL to 800 g pellet of mouse mammary gland. Each point represents mean of duplicate observations.

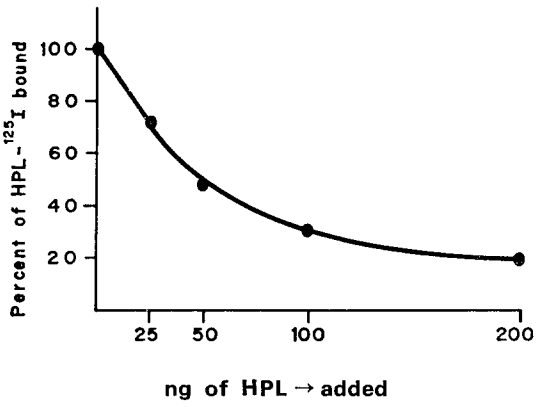


Fig. 4. Binding of ^{125}I-HPL to 800 g pellet of mammary gland tissue in presence of various amounts of unlabelled HPL. Each point represents mean of duplicate observations.

Figure 4 illustrates the competition between labelled and unlabelled HPL. Increasing concentrations of unlabelled HPL (from 25 to 200 ng) resulted in a competitive inhibition of the binding of labelled hormone. At a concentration of 200 ng of unlabelled hormone the binding of labelled ^{125}I-HPL is as low as 20 %, as compared to 100 % binding where no unlabelled HPL is added.

The results of the differential centrifugation studies designed to ascertain whether the 800 *g* supernatant contained hormone binding components revealed that the 10,000 and 100,000 *g* pellets, operationally defined as mitochondrial and microsomal fractions respectively, also showed binding (Table 1). In fact, both of these fractions showed significantly higher binding than that of 800 *g* pellet.

Figure 5 illustrates the effect of enzyme digestion on the binding capacity of 800 and 10,000 *g* pellets. Incubation with degradative enzyme, like chymotrypsin greatly reduced the binding capacity of 800 as well as 10,000 *g* pellets of mammary gland as compared to that of control. Unlike chymotrypsin, collagenase significantly enhanced the binding capacity of 800 and 10,000 *g* pellets to ^{125}I-HPL. Recently Saxena and Haour [37] reported that treatment of corpora lutea receptor with phospholipase increased the binding of labelled HCG. However, no appreciable change was observed with trypsin and proteinase. These results suggest that the binding components of 800 and 10,000 *g* pellets are at least partly

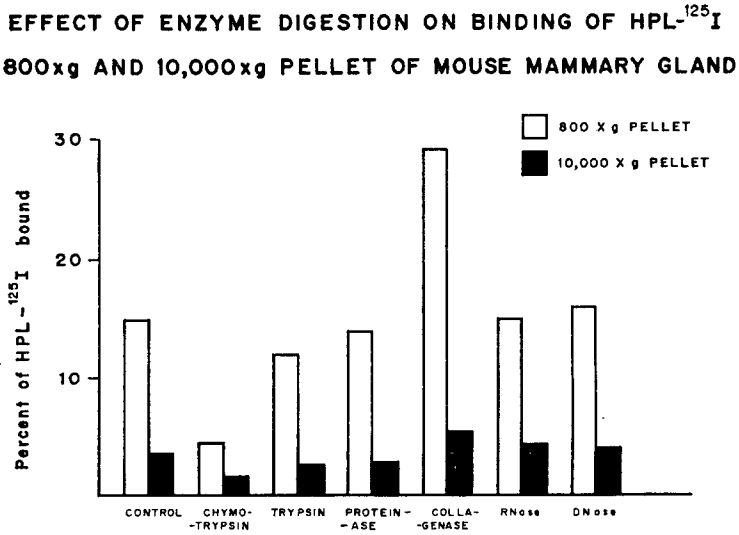


Fig. 5. Effect of enzyme digestion on binding of ^{125}I-HPL to 800 g and 10,000 g pellets of mouse mammary gland. Each histogram is a mean of three observations.

protein in nature. There was no effect of RNase and DNase on the binding capacity of both the pellets indicating that the binding components are not nucleic acids.

Table 1. *Subcellular localization of* ^{125}*I-HPL binding components of mammary gland*

Sedimentable fraction	Percentage of initial ^{125}I-HPL bound to pellet
800 *g* pellet	3·38 ± 0·249
10,000 *g* pellet	6·54 ± 0·419
100,000 *g* pellet	10.16 ± 0.642

Results are mean of 4 observations with S.E. of mean.
Levels of significance

Between 800 *g* pellet and 10,000 *g* pellet. } *P* less than 0.001.
Between 800 *g* pellet and 100,000 *g* pellet }

It was considered of interest to study the effect of theophylline on binding of ^{125}I-HPL to 800 *g* pellet (Table 2). The binding was significantly increased with the addition of 1·2 and 1·6 mg of theophylline to the homogenates, while a lower amount of 0·4 mg had no detectable effect.

Table 2. *Effect of theophylline on binding of* ^{125}*I-HPL to* 800 *g pellet of mammary gland*

Theophylline	Percentage of total ^{125}I-HPL bound	Percentage increase over control
Control (nil)	3·666	0
0·4 mg	3·680	0
1·2 mg	6·025	64
1·6 mg	8·092	100

Results are mean of duplicates.

It may be observed from Table 3 that ^{125}I-HPL binds to 800 *g* pellet of mouse mammary gland to a greater extent than any of the other hormones studied. The binding with ^{125}I-HLH and ^{125}I-HFSH is not significant.

After the initial studies on binding characteristics it was of primary interest to compare the binding capacity of mammary gland of cancer susceptible and resistant strains of mice. It may be observed from Table 4 that 800 **g** pellet of mammary gland of C3H (Jax) mice binds ^{125}I-HPL to a significantly greater extent than that of C57 BL mice.

Table 3. *Binding of* ^{125}*I-HPL,* ^{125}*I-HCG,* ^{125}*I-HLH and* ^{125}*I-HFSH to* 800 *g pellet of mouse mammary gland*

Hormone	Percentage of total hormone bound
^{125}I-HPL	4·180 ± 0·104
^{125}I-HCH	1·015 ± 0·01
^{125}I-HLH	< 0·5
^{125}I-HFSH	< 0·5

Results are mean of 4 observations with S.E. of mean.

Table 4. *Binding of* ^{125}*I-HPL to* 800 *g pellet of mammary gland of C3H (Jax) and C57 BL strains of mice*

Strains	Percentage of total ^{125}I-HPL bound per mg of protein
C3H (Jax)	4·178 ± 0·13*
C57 BL	0·71 ± 0·02

Results are mean of 4 observations with S.E. of mean.
*Denotes statistical significance when *P* value is < 0·05.

DISCUSSION

The results of the present study have shown a few interesting characteristics of HPL binding to mouse mammary gland. The susceptibility of binding components to proteolytic enzymes and the increased binding of the hormone in the presence of proteolytic enzyme inhibitor (TAME) are indicative of the proteinaceous nature of the binding material. As RNase and DNase had no effect on binding of 800 and 10,000 *g* pellets, it is unlikely that nucleic acids are directly involved.

It is widely believed that protein hormones do not enter the target cell but interact with the cell membrane. However, the present results on binding to subcellular fractions and recent studies with HCG [27], LH [25, 38], FSH [39] and HPL [28] suggest that the hormone may bind with intracellular components like nuclei, mitochondria and microsomes besides the cell membrane. However, it is also possible that the distribution of binding of labelled HPL in the mitochondrial and microsomal fractions may be due to cell membrane which could have been disrupted during homogenization in a variety of sizes and subsequently is distributed through all fractions during differential centrifugation.

We have observed (Table 1, Fig. 5) that binding of ^{125}I-HPL to 800 *g* pellet increases

considerably if the 800 *g* pellet was separated at the beginning of the experiment (Fig. 5) instead of using the whole homogenate and then separating the 800 *g* pellet at the end of the experiment (Table 1). This is probably due to the fact that proteolytic enzymes present in the 800 *g* supernatant will decrease the binding of hormone when whole homogenate is incubated. This observation is also supported by our experiments (Fig. 3) that 800 *g* pellet bound more hormone in the presence of proteolytic enzyme inhibitor, TAME.

Theophylline increases the binding capacity of 800 *g* pellet. Theophylline is known to act as an inhibitor to the enzyme phosphodiesterase, itself an inactivator of cyclic 3′, 5′-AMP by catalysing its hydrolysis to 5′-AMP. Since it is well established that cyclic AMP plays a major role as a second messenger to hormone action [40], it seems possible that theophylline affects the levels of cyclic AMP enhancing binding capacity either by new synthesis of hormone receptors or by stabilizing the existing receptors. Recently Rao [41] has reported that binding of labelled-HCG to bovine corpora lutea was considerably increased in the presence of cylic nucleotides.

Our observations on *in vitro* binding of labelled HPL to mouse mammary gland do not in themselves provide evidence for physiological interaction of the hormone with the tissue. However, if binding is considered to be the initial step of mechanism of action of hormone then the significantly greater binding of HPL to mammary gland of mice of the susceptible C3H (Jax) strain as compared to that of the resistant C57 BL strain may be correlated to the differential susceptibility of the mammary gland to the development of breast cancer. In fact, Nagasawa Yanai, Iwahashi, Fujimoto and Kuretani have observed that C3H/He mice showed a more marked mammary development in response to pituitary mammotropins than C57 BL/6 mice [42]. Yanai and Nagasawa have also reported that C3H/He mice are superior to C57 BL/6 mice in mammary development and lactation [43]. Also noteworthy is the fact that Sinha, Selby, Lewis and Vanderlaan have observed lower serum prolactin levels in mice of C3H (Jax) than that in C57 BL strain of mice [44]. Present observations on higher binding of HPL to 800 *g* pellet of mammary gland of C3H (Jax) strain mice encourages further study of the prolactin binding capacity of the mammary gland of susceptible as well as of resistant strains of mice.

REFERENCES

1. K. Kohmoto and H. A. Bern, Demonstration of mammotropic activity of the mouse placentae in organ culture and by transplantation. *J. Endocr.* **48,** 99 (1970).
2. C. Desjardins, M. J. Paape and H. A. Tucker, Contribution of pregnancy, fetuses, fetal placentas and deciduomas to mammary gland and uterine development. *Endocrinology* **83,** 907 (1968).
3. W. G. Kinzey, Hormonal activity of the rat placenta in the absence of dietary protein. *Endocrinology* **82,** 266 (1968).
4. W. R. Lyons, Hormonal synergism in mammary growth. *Proc. roy. Soc. B* **149,** 303 (1958).
5. D. L. Mathies, Studies of the luteotropic and mammotropic factor found in trophoblast and maternal peripheral blood of the rat at midpregnancy. *Anat. Record* **159,** 55 (1967).
6. D. L. Mathies, Rapid assay for the lactogenic activity of rat chorionic mammotropin. *Proc. Soc. expt. Biol. (N.Y.)* **127,** 1126 (1968).
7. D. L. Mathies and W. R. Lyons, Luteotrophic and luteolytic effects of rat chorionic mammotropin. *Proc. Soc. expt. Biol. (N.Y.)* **136,** 520 (1971).
8. J. Shani (Mishkinsky), L. Zanbelman, K. Khazen and F. G. Sulman, Mammotrophic and prolactin-like effects of rat and human placentae and amniotic fluid. *J. Endocr.* **46,** 15 (1970).
9. H. L. Buttle and I. A. Forsyth, Prolactin concentration and lactogenic activity in the plasma of pregnant and lactating goats. *J. Endocr.* **51,** xxxiii (1971).
10. H. L. Buttle, I. A. Forsyth and G. S. Knaggs, Plasma prolactin measured by radioimmunoassay and bioassay in pregnant and lactating goats and the occurrence of a placental lactogen. *J. Endocr.* **53,** 483 (1972).
11. H. G. Friesen, Biosynthesis of placental proteins and placental lactogen. *Endocrinology* **83,** 744 (1968).

12. H. G. Friesen, S. Suwa and P. Pare, Synthesis and separation of placental lactogen and other proteins by the placenta. *Recent Progr. Hormone Res.* **25,** 161 (1969).
13. D. B. Grant, S. L. Kaplan and M. M. Grumbach, Studies on a monkey placental protein with immunochemical similarity to human growth hormone and human chorionic somatomammotrophin. *Acta Endocr.* (*Kbh.*) **63,** 736 (1970).
14. S. L. Kaplan and M. M. Grumbach, Studies of a human and simian placental hormone with growth hormone-like and prolactin-like activities. *J. clin. Endocr. Metab.* **24,** 80 (1964).
15. B. N. Saxena, Protein-polypeptide hormones of the human placenta. *Vitam. u. Horm.* **29,** 95 (1971).
16. E. C. Amaroso and D. G. Porter, Anterior pituitary function in pregnancy. In *The Pituitary Gland.* (Edited by G. W. Harris and B. T. Donavan) Vol. II, p. 364, Butterworth, London (1966).
17. A. T. Cowie and J. S. Tindal, *The Physiology of Lactation,* p. 53, Edward Arnold, London (1971).
18. R. W. Turkington and Y. J. Topper, Stimulation of casein synthesis and histological development of mammary gland by human placental lactogen *in vitro. Endocrinology* **79,** 175 (1966).
19. R. W. Turkington, Induction of milk protein synthesis by placental lactogen and prolactin *in vitro. Endocrinology* **82,** 575 (1968).
20. L. Fould, Mammary tumors in hybrid mice. Growth and progression of spontaneous tumors. *Brit. J. Cancer* **3,** 345 (1949).
21. R. Yanai and H. Nagasawa, Enhancement by human placental lactogen of mammary hyperplastic nodules in ovariectomized mice. *Cancer Res.* **33,** 1642 (1973).
22. I. Pastan, J. Roth and V. Macohia, Binding of hormone to tissue: The first step in polypeptide hormone action. *Proc. nat. Acad. Sci.* (*Wash.*) **56,** 1802 (1966).
23. B. P. Schimmer, K. Ueda and G. H. Sato, Site of action of adrenocorticotropic hormone (ACTH) in adrenal cell cultures. *Biochem. biophys. Res. Commun.* **32,** 806 (1968).
24. K. J. Calt, M. L. Dufau and T. Tsuruhara, Studies on radioligand-receptor assay system for luteinizing hormone and chorionic gonadotropin. *J. clin. Endocr.* **32,** 860 (1971).
25. D. M. De Krester, K. J. Calt and C. A. Paulsen, Studies on the *in vitro* testicular binding of iodinated luteinizing hormone in rats. *Endocrinology* **88,** 332 (1971).
26. M. H. Rodbell, M. J. Krans, S. L. Pohl and L. Birnbaumer, The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. *J. biol. Chem.* **246,** 1861 (1971).
27. B. J. Danzo, A. R. Midgley, Jr. and L. J. Kleinsmith, Human chorionic gonadotropin binding to rat ovarian tissue in *vitro. Proc. Soc. exp. Biol.* (*N.Y.*) **139,** 88 (1972).
28. S. B. Moodbidri, A. R. Sheth and S. S. Rao, *In vitro* binding of radioiodinated human placental lactogen to buffalo corpus luteum. *J. Reprod. Fertil.* **35,** 453 (1973).
29. N. A. Sheth, M. M. Wagle, S. V. Bhide and K. J. Ranadive, Studies on sequential metabolic alteration in spontaneous mammary carcinogenesis. *Br. J. Cancer* **21,** 228 (1967).
30. N. A. Sheth, S. V. Bhide and K. J. Ranadive, Xanthine oxidase activity in progressive spontaneous mammary carcinogenesis. *Br. J. Cancer* **22,** 833 (1968).
31. N. A. Sheth, S. V. Bhide and K. J. Ranadive, Incorporation of ^{32}P into nucleic acids in mammary tissue of mice susceptible and resistant to breast cancer. *J. nat. Cancer Inst.* **46,** 731 (1971).
32. N. A. Sheth and S. V. Bhide, Comparative study of protein biosynthesis in mice susceptible and resistant to breast cancer. *J. nat. Cancer Inst.* **51,** 1977 (1973).
33. K. A. Karande, N. A. Sheth and K. J. Ranadive, Follicle stimulating hormone content of pituitary gland of intact and ovariectomized mice of different strains. *J. Endocr.* **48,** 457 (1970).

34. F. C. Greenwood, W. M. Hunter and J. S. Glover, The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89,** 114 (1963).
35. A. R. Midgley, Jr., Radioimmunoassay, A method for human chorionic gonadotropin and human luteinizing hormone. *Endocrinology* **79,** 10 (1966).
36. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
37. B. B. Saxena and F. Haour, Characterization and purification of the gonadotrophin receptor of the corpus luteum. Paper presented at *Symposium on Advances in Chemistry, Biology and Immunology of Gonadotrophins*,, 21–27 Oct., 1973, Bangalore, India.
38. P. Caulson, T. C. Liu, P. Morris and J. Gorski, Interaction of LH with ovary. In *Gonadotrophins*. Edited by B. B. Saxena, C. G. Beling and H. M. Grandy, p. 227. Wieley Interscience, USA. (1972).
39. A. R. Means and J. Vaitukaitis, Peptide hormone "receptor" specific binding of ^{3}H-FSH to testis. *Endocrinology* **90,** 39 (1972).
40. G. A. Robison, R. W. Butcher and E. W. Sutherland, Cyclic AMP and hormone action. In *Cyclic AMP*. (Edited by G. A. Robison, R. W. Butcher and E. W. Sutherland) Chapter 2, p. 17. Academic Press, New York and London (1971).
41. Ch. V. Rao, Receptor for prostaglandins and gonadotrophins in the cell membranes of bovine corpora lutea. Paper presented at *Symposium on Advances in Chemistry, Biology and Immunology of Gonadotrophins*, 21–27 Oct. 1973, Bangalore, India.
42. H. Nagasawa, R. Yanai, H. Iwahashi, M. Fujmoto and K. Kuretani, Difference in mammary gland susceptibility to prolactin between a high and low mammary tumor strain of mice. *Endocr. Jap.* **14,** 351 (1967).
43. R. Yanai and H. Nagasawa, Pituitary prolactin and growth hormone levels during different reproductive status in mice with a high or a low lactational performance. *Hormones and Behaviour*, Vol. II, p. 73. Academic Press, New York (1971).
44. Y. N. Sinha, F. W. Selby, U. J. Lewis and W. P. Vanderlaah, Studies of prolactin secretion in mice by homologous radioimmunoassay. *Endocrinology* **91,** 1045 (1972).

Europ. J. Cancer Vol. 10, pp. 661–666. Pergamon Press 1974. Printed in Great Britain.

Immune Manipulation by BCG Administered before or after Cyclophosphamide for Chemo-Immunotherapy of L1210 Leukaemia

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Abstract—1. *BCG (1 mg/mouse) administered i.v. on day 5 after chemotherapy by cyclophosphamide (CPM), given itself at the dose of 80 or 134 or 403 mg/kg one day after the inoculation of L1210 leukaemia 10^3 cells, increases the effect of the non-optimal doses: i.e. 80 mg/kg, which is insufficient to cure mice, and 403 mg/kg, which is toxic. It does not change the effect of the optimal dose of CPM (134 mg/kg) which cures most of the mice.*

2. *BCG (1 mg/mouse i.v.) given on day 15 before CPM decreases the curative effect of the latter at the dose of 134 mg/kg.*

These results suggest that: (a) BCG given after chemotherapy is not only able to eradicate the residual disease, but can decrease chemotherapy toxicity; (b) BCG given before an optimal dose of chemotherapy may decrease its antileukaemic effect, via the enhancement of the chemotherapy immunodepressive action, as demonstrated by skin graft experiments; this deterioration of the effect of chemotherapy by immunodepression was also suggested when the combination of CPM and ATS was used.

INTRODUCTION

WE HAVE shown previously that BCG administered alone exerts an antitumour effect on subcutaneously grafted L1210 leukaemia, provided the number of grafted tumour cells is $\leqslant 10^5$. This effect is moderate when BCG is administered 1 – 4 days after injecting the leukaemia cells (immunotherapy) and intensive when it is given 14 days before (immunoprophylaxis) [1, 2]. The action of BCG combined with irradiated L1210 leukaemia cells has a more efficient antitumour effect in both conditions than BCG alone [1, 2].

The curative action of BCG alone or combined with irradiated leukaemic cells is also attained when a larger number of leukaemic cells is inoculated and subsequently reduced by chemotherapy [3, 4].

Hence, we proposed the use of active immunotherapy in clinical practice for the eradication of the residual imperceptible disease left after chemotherapy (or other means of treatment) of the overt tumour mass.

This was the basis of our first clinical trial of immunotherapy in patients with active lymphoid leukaemia. The sequence of our protocol was as follows: (a) chemotherapy to induce remission; (b) complementary, cell-reducing, systemic chemotherapy and meningeal chemoradiotherapy; (c) active immunotherapy (comprising BCG and/or irradiated leukaemic cells). The results obtained in this randomized trial demonstrated the beneficial effect of active

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immunotherapy [5] (7 patients out of 20 are still in remission 7–10 years after the end of chemotherapy compared to none in the non-immunotherapy control group). The overall results obtained so far in 200 patients with ALL are excellent as more than 35 % of the patients of all ages are alive in remission at 5 years [6], and more than 90 % of the patients aged less than 15 years with the so-called "micro-lymphoblastic" type [7], are alive at five years in perfect health both clinically and haematologically.

Other trials have confirmed the action of immunotherapy after chemo-radiotherapy in patients with terminal leukaemic lympho-sarcoma (BCG and irradiated tumour cells) [8], in patients with acute myeloid leukaemia (BCG alone) [9], and its adjuvant action after surgery in melanoma [10, 11].

In these trials, immunotherapy (BCG alone or combined with irradiated tumour cells) was given following the completion of chemo-therapy and after an interval of time which had been demonstrated, in a sarcoma model system in animals, to be necessary for active immuno-therapy to be effective [12].

In other trials, phases of immunotherapy were interspersed with courses of chemo-therapy: i.e. in the treatment of chronic myeloid leukaemia [13], non-terminal lym-phomas [14], and acute myeloid leukaemia [15].

In interspersed regimens, it is questionable whether immunotherapy has any effect on the cell kinetics of lymphocytes. The entry of many lymphocytes into the cycle, especially in the thymus in which many pyronophilic cells and mitoses can be seen after an intravenous injection of BCG in mice [16], may render the subjects more sensitive to the immunosup-pressive effect of chemotherapy.

The purpose of the experiment reported in this paper is to compare the effect of the sequential administration of BCG followed by cyclophosphamide (CPM) with that of CPM followed by BCG.

MATERIAL AND METHODS

L1210 leukaemia and recipients

L1210 leukaemia has been chosen because our first experiments which demonstrated the action of active immunotherapy were made using this model system [1, 2]. One thousand living cells were inoculated i.v. on day 0 in all groups; (C57Bl/6 × DBA/2) F1 mice aged 2½ months were the recipients.

BCG

Fresh Pasteur BCG preparation, which is the only constantly active among ten preparations [17] was injected i.v.; [this is the only route of administration that gives the best results [17], at the dose of 1 mg (7×10^6 living bacteria) per mouse].

Cyclophosphamide

CPM was given intraperitoneally (i.p.) on day 1 after leukaemia inoculation in three different doses: 80 mg/kg, which will delay mortality of leukaemic animals but is unable to cure any of them; 134 mg/kg, which is the optimal non-toxic dose and is able to cure 88 % of the leukaemic mice; and 403 mg/kg, which is able to cure some mice, but kills 65 % of these animals from bone marrow (and lymphoid ?) toxicity.

Antithymocyte serum (ATS) was used in a control experiment to check if immunosuppression is able to decrease the antileukaemic effect of 134 mg/kg of CPM. The preparation used consists of rabbit anti-mouse thymocytes (Microbiological Associates, Bethesda, USA) and it was given i.p. at the dose of 0·25 ml/mouse 8, 6, 3 and 1 day(s) before the inoculation of leukaemia cells.

Experiments

Five experiments were conducted:

(1) In the first, the immunoprevention and the immunotherapeutic effect of 1 mg of BCG were tested in mice inoculated with BCG alone.

(2) In the second, third and fourth experiments, CPM given alone, CPM preceded by BCG injected at day-14, and CPM followed by BCG injected at day +6 were compared: the dose of CPM was 80 mg/kg in Experiment 2, 134 mg/kg in Experiment 3, and 403 mg kg in Experiment 4 see Fig. 2, 3, and 4).

(3) In the fifth experiment, the combination of ATS (given according to the conditions described above) with 134 mg/kg of CPM was compared with CPM given alone at that dose.

(4) The mortality of the mice was recorded daily, autopsies made, and the differences between the groups in the same experiment were analysed by the χ^2 test. Each group contained 16 to 20 mice.

RESULTS

The first experiment confirms previously published data [1, 2]: 1 mg i.v. BCG, not combined with chemotherapy, exerts a significant immunoprophylactic effect on L1210 leukaemia

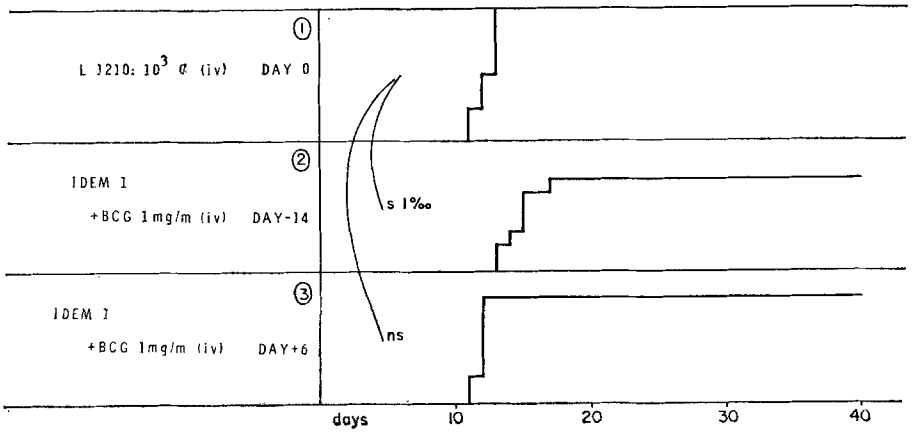


Fig. 1. *Significant prophylactic effect of BCG administered alone* (1 mg *i.v./mouse*) *on L*1210 *leukaemia* (10^3 *cells*)*; the slight immunotherapeutic action is not significant.*

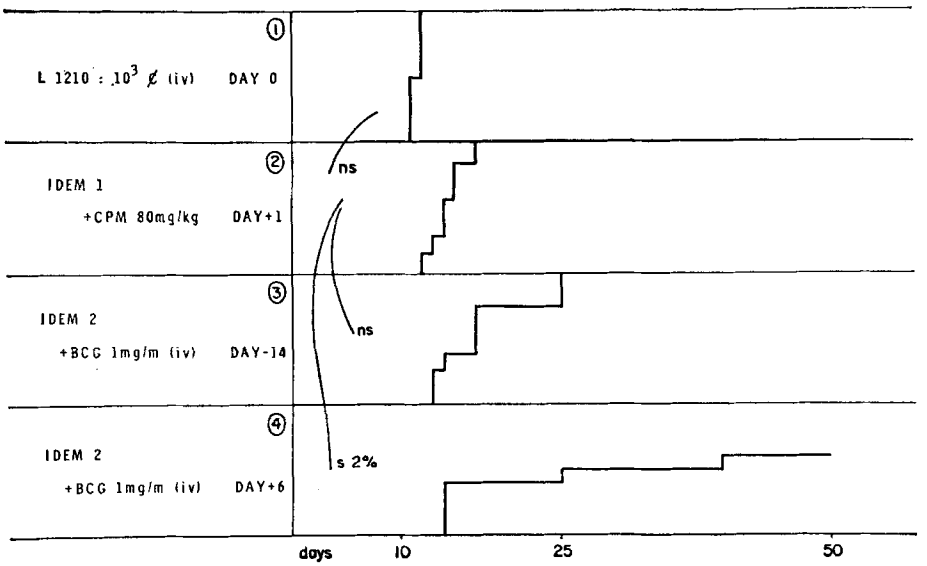


Fig. 2. *Immunotherapeutic effect of BCG administered* 5 *days after injection of* 80 *mg/kg of CPM.*

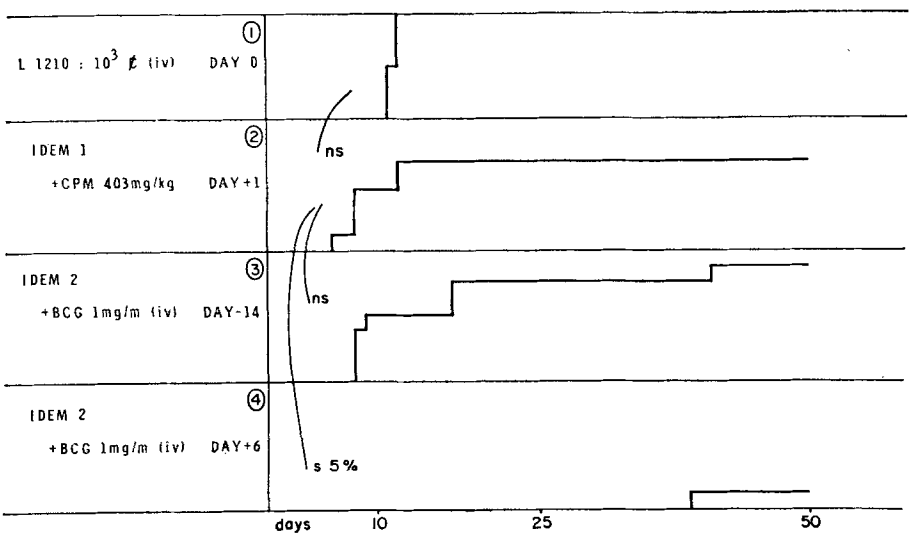


Fig. 3. *Immunotherapeutic effect of BCG given* 5 *days after the injection of* 403 *mg/kg of CPM and its protective effect against early bone marrow* (*and lymphoid?*) *toxicity.*

and a slight but not significant immunotherapeutic action (Fig. 1).

The second experiment (Fig. 2) shows that 80 mg/kg of CPM administered on day 1 after L1210 cell inoculation exerts a moderate but not significant antileukaemic effect: it does not cure any animals. One mg BCG injected on day +6 considerably increases the action of CPM, curing 8 mice out of 20. Hence, the immunotherapeutic effect of BCG is enhanced by a preceding dose of a moderately active chemotherapy. BCG injected before CPM, on day −14 before leukaemia inoculation, does not change the effect of chemotherapy.

The third experiment (Fig. 3) shows that the largest doses of CPM (403 mg/kg) has a double effect: it cures a third of the mice but it kills two thirds of them from bone marrow (and lymphoid ?) toxicity. BCG administered before the CPM tends to decrease the cure rate and does not reduce the toxicity mortality. However, this experiment exhibits a double effect of BCG given after CPM: an immunotherapeutic effect and, which may be more important, a protective effect against early mortality from bone marrow (and lymphoid ?) aplasia.

The fourth experiment deals with the use of the optimal dose of CPM. One hundred and thirty-four mg/kg CPM cures 16 out of 18 mice. As a consequence there can be no demonstrable effect of BCG administered after CPM. But the interesting observation is the deterioration of the antileukaemic effect of CPM by BCG injected before the chemotherapy (Fig. 4).

As our working hypothesis was that BCG, by pushing the lymphocytes into the cycle, could increase the immunodepressive effect of CPM because this drug is a cycle-dependent agent [18–20], we have studied the effect of the combination of ATS and CPM. While ATS alone does not affect the survival of the controls, it can be seen in Fig. 5 that the immunosuppression induced by ATS reduces the antileukaemic effect of 134 mg/kg of CPM.

DISCUSSION

In conclusion, the experimental model used in this work clearly suggests that: (1) BCG is efficient: (*a*) as an immunotherapeutic agent, even when injected alone after a suboptimal dose of CPM (80 mg/kg); (*b*) as an immunotherapeutic and protective agent against bone marrow and possible lymphoid toxicity when injected after a high and toxic dose of chemotherapy (403 mg/kg), which has also been shown in another experiment [21]; (2) on the contrary, when injected before CPM, it does not modify its therapeutic and toxic effects when the doses of CPM are suboptimal or toxic, but it reduces the antileukaemic action of the optimal dose of CPM. Our interpretation of this effect is that BCG enhances the immunosuppression induced by CPM, (probably by pushing the lymphocytes into the cycle): this hypothesis has been proven by the skin graft experiments [22]; similar data have also been observed with the hemolytic plaque forming cell test and procarbazine [23]; and the fact that the antileukaemic effect of the optimal dose of CPM is decreased by immuno-

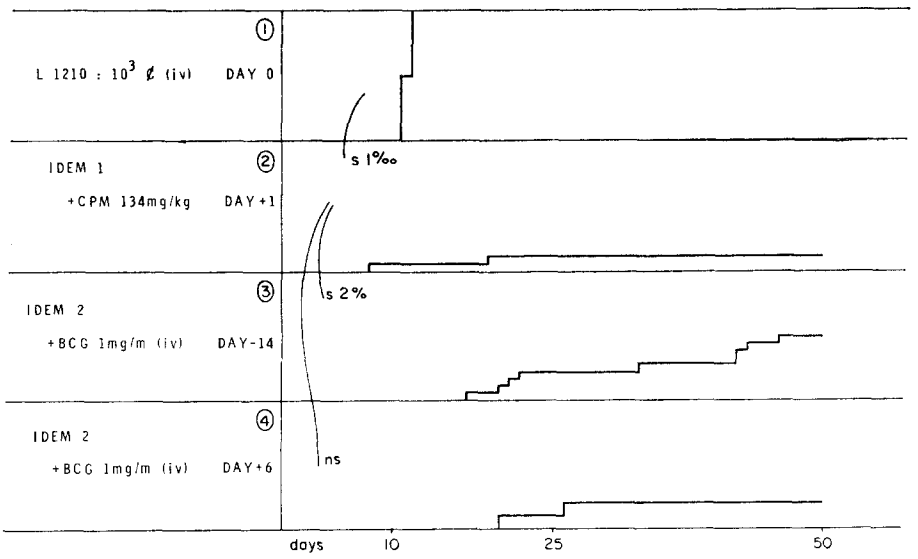


Fig. 4. Deterioration by BCG given before 134 mg/kg of CPM on its antileukaemic effect.

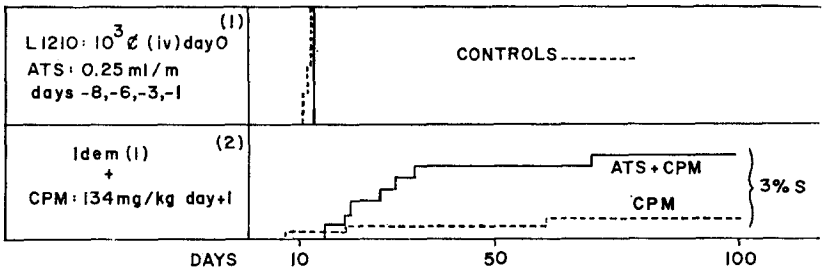


Fig. 5. Deterioration by ATS immunodepression of the antileukaemic effect of 134 mg/kg of CPM.

suppression has been clearly demonstrated by an experiment in which ATS and CPM were combined.

Hence these results favour the use of the following sequence in clinical therapy: (1) cell reducing chemotherapy; (2) active immunotherapy for the eradication of tumours. The experiments stress the possible risks from using interspersed combinations of chemotherapy and immunotherapy, which may not obtain the maximal effect or indeed the same effect as the recommended sequence. This conclusion is valuable for chemotherapy compounds which are immunosuppressive but may not be applicable to those compounds which are not [24].

REFERENCES

1. G. Mathé, Immunothérapie active de la leucémie L1210 appliquée après la greffe tumorale. *Rev. franc. Etud. clin. biol.* **9,** 881 (1968).
2. G. Mathé, P. Pouillart and F. Lapeyraque, Active immunotherapy of L1210 leukaemia applied after the graft of tumour cells. *Brit. J. Cancer* **23,** 814 (1969).
3. G. Mathé, Active immunotherapy. *Adv. Cancer Res.* **14,** 1 (1971).
4. J. L. Amiel and M. Bérardet, An experimental model of active immunotherapy preceded by cytoreductive chemotherapy. *Europ. J. Cancer* **6,** 557 (1970).
5. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat and F. de Vassal, Active immunotherapy for acute lymphoid leukaemia. *Lancet* **i,** 697 (1969).
6. G. Mathé, J. L. Amiel, L. Schwarzenberg, M. Hayat, P. Pouillart, M. Schneider, A. Cattan, J. R. Schlumberger, F. de Vassal, M. Musset and J. L. Misset, Dix ans d'immunothérapie active des leucémies aiguës et des lymphosarcomes leucémiques. Etude de 200 cas. *Nouv. Presse med.*, (In press) (1974).
7. G. Mathé, P. Pouillart, M. Sterescu, J. L. Amiel, L. Schwarzenberg, M. Schneider, M. Hayat, F. de Vassal, C. Jasmin and M. Lafleur, Subdivision of classical varieties of acute leukemia: correlation with prognosis and cure expectancy. *Europ. J. clin. biol. Res.* **16,** 554 (1971).
8. G. Mathé, P. Pouillart, L. Schwarzenberg, M. Hayat, J. L. Amiel and F. de Vassal, Leukaemic conversion of non-Hodgkin's malignant lymphomas. *Brit. J. Cancer*, (In press) (1974).
9. W. R. Vogler and Y. K. Chan, Effect of Bacillus Calmette Guérin (BCG) in prolongation of remissions in acute myeloblastic leukemia (AML). *Proc. Am. Ass. Cancer Res.* **15,** 164 (Abstract No. 723) (1974).
10. A. Z. Bluming, C. L. Vogel, J. L. Ziegler, N. Mody and G. Kamya, Immunological effects of BCG in patients with malignant melanoma. A comparison of two modes of administration. *Ann. intern. Med.* **76,** 405 (1972).
11. J. U. Gutterman, C. McBride, E. J. Freireich, G. Mavligit, E. Frei III and E. M. Hersh. Active immunotherapy with BCG for recurrent malignant melanoma. *Lancet* **i,** 1208 (1973).
12. G. A. Currie and K. D. Bagshawe, Active immunotherapy with Corynebacterium parvum and chemotherapy in murine fibrosarcomas. *Br. med. J.* **1,** 541 (1970).

13. J. E. Sokal and C. W. Aungst, Immunization with cultured cell–BCG mixtures. In *Investigation and Stimulation of Immunity in Cancer Patients.* (Edited by G. Mathé and R. Weiner,) Vol. I, Springer Verlag, CNRS, Paris (1974).
14. J. E. Sokal, C. W. Aungst and M. Snyderman, Prolongation of remission in stage I and II lymphoma by BCG vaccination. *Proc. Am. Ass. Cancer Res.* **15,** 13 (abstract no. 51) (1974).
15. R. Powles, H. E. M. Kay, T. J. McElwain, P. Alexander, D. Crowther, G. Hamilton-Fairley and M. Pike, Immunotherapy of acute myeloblastic leukaemia in man. In *Investigation and Stimulation of Immunity in Cancer Patients.* (Edited by G. Mathé and R. Weiner,) Vol. I, Springer Verlag; CNRS, Paris (1974).
16. A. Khalil, H. Rappaport, C. Bourut, O. Halle-Pannenko and G. Mathé, Tissue reactions to intravenous and subcutaneous BCG injections; their significance for cancer immunotherapy. *Biomedicine* (In press) (1974).
17. G. Mathé, O. Halle-Pannenko and C. Bourut, BCG in cancer immunotherapy II. Results obtained with various BCG preparations in a screening study for systemic adjuvants applicable to cancer immunoprophylaxis or immunotherapy. *Nat. Cancer Inst. Monogr.* **39,** 109 (1973).
18. W. R. Bruce, The action of chemotherapeutic agents at the cellular level and the effect of these agents on hematopoietic and leukaemic tissue. *Canad. Cancer Conf.* **7,** 53 (1966).
19. L. M. van Putten and P. Lelieveld, Factors determining cell killing by chemotherapeutic agents *in vivo*.—I. Cyclophosphamide. *Europ. J. Cancer* **6,** 213 (1970).
20. L. M. van Putten and P. Lelieveld. Factors determining cell killing by chemotherapeutic agents *in vivo*—II. Melphalan, chlorambucil and nitrogen mustard. *Europ. J. Cancer* **7,** 11 (1971).
21. G. Mathé, Prevention of chemotherapy complications: time, toxicity, pharmacokinetic, pharmacodynamic and logistic factors. In *Complications of Cancer Chemotherapy.* (Edited by G. Mathé and R. K. Oldham,) Vol. I. Springer Verlag, Heidelberg (1974).
22. G. Mathé, O. Halle-Pannenko and C. Bourut, Potentiation of a cyclophosphamide (CPM) induced immunodepression by the administration of BCG. *Transplant. Proc.* (In press) (1974).
23. 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. franc. Etud. clin. biol.* **14,** 912 (1969).
24. W. Pearson, S. D. Chaparas and M. A. Chirigos, Effect of dose and route of Bacillus Calmetter Guérin in chemo-immunostimulation therapy of a murine leukemia. *Cancer Res.* **33,** 1845 (1973).

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Cytotoxic Effect *In Vitro* and Tumour Volume Reducton *In Vivo* induced by Chemotherapeutic Agents*

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Abstract—*By a comparison of* in vitro *and* in vivo *effectiveness of cytostatic agents on a transplantable mouse osteosarcoma, a number of drugs were identified for which the* in vitro *response is not a good prediction for the* in vivo *response. For a number of such discrepancies, explanations have been identified or tentatively been put forward, namely:*

(1) *absence* in vitro *of drug metabolizing systems which are effective* in vivo.

(2) *too low tumour drug levels reached by safe drug doses;*

(3) *too short duration of effective drug levels after single dose* in vivo.

For a number of drugs, unidentified factors cause such discrepancies. However, there is another group of widely used cytostatic agents for which an in vitro *test may give a good prediction of overall tumour response and further studies seem to confirm this for other types of tumours.*

THE VARIATION in effectiveness of different chemotherapeutic agents on various tumours is related partly to the variation in inherent sensitivity of the tumour cells to these agents. It must be noted, however, that the definition of "active" drug *in vivo* is not necessarily related to the acute cell killing capacity of the drug itself or of its metabolites, but to the resultant of several effects leading to the overall result of increase in life span or of decrease in tumoral mass. To break through the problem posed by many variables it is necessary to study independently each of the factors involved.

In this paper the sensitivity *in vitro* of an experimental tumour is compared with the acute effect induced on tumour volume *in vivo* by equitoxic doses of drugs. An *in vitro* method has been developed and internally tested for reproducibility in an attempt at characterization of cancer cells from different tumours by a chemotherapeutic fingerprint [1]. This permits evaluation of the degree to which the *in vitro* sensitivity of a drug on cells of a tumour may be used to predict its effectiveness for therapy. In this way discrepancies are detected and interpreted in the light of our knowledge on activation or inactivation of drugs *in vivo* and on the differences in cellular drug exposure *in vivo* and *in vitro* depending on the administration schedule. Although our model tumour is much more homogeneous and uniform than human tumours, a study of this kind may at least indicate a number of drugs for which a simple prediction cannot be expected and for which other techniques [2] should be tried by which a better prediction may be obtained.

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MATERIAL AND METHODS

Tumour

The osteosarcoma C22 LR was induced in 1957 by internal irradiation from Strontium-90 in a female (C_{57}Bl/Rij × CBA/Rij) F_1 hybrid

mouse. The tumour is non-antigenic in the hybrid mice in which it originated and has been serially transplanted or stored in liquid nitrogen. Its properties have been described [3]. The doubling time is from 1·8 to 2·2 days. For the studies described here, use was made of passages 76 through 81, derived from a large quantity of cell suspensions from passage 75 which was stored in liquid nitrogen.

Determination of the tumour volume reduction ratio (TVR)

A suspension containing 10^6 C22 LR cells in 0·1 ml was injected s.c. in each flank of several mice. After 10 days, tumours were measured in three dimensions by caliper and the animals randomized. Groups of five mice were then treated by an LD_{10} dose of each agent, in a single subcutaneous injection in saline. Insoluble compounds were suspended in 2% carboxymethylcellulose. Four days later, tumours were measured again and the volume reduction factor was calculated as the relative volume of the treated over the control tumours at that time. Standard errors are of the order of 20% of the mean and the 95% confidence limits are ±52%. Experience has shown that a tumour volume reduction factor over 0·50 indicates only temporary arrest of growth; moderate cytostatic effectiveness (+) is associated with a TVR between 0·50 and 0·25 and a factor below 0·25 indicates good effectiveness (++) of the agent.

Determination of the chemotherapeutic fingerprint in vitro

Fragments of C22 LR tumour were minced by two sharp blades to prepare fragments about 1 mm in size. These were explanted in a Rose chamber under perforated cellophane [4, 5] and fed with medium 199 buffered by HEPES and supplemented with 20% calf serum. After 5 days incubation at 36·5 ± 0·5°C, cells started to migrate from the explant in a typical outgrowth. As soon as the migration started, microscopic fields were marked on the outside of the coverslip by india ink and pictures were taken with phase contrast optics. Cultures were then randomized and exposed to tenfold serially increasing concentrations of the drugs. After 24 and 48 hr of incubation in presence of the drug the microscopic fields were photographed again.

Results were recorded as the concentration of drug able to induce cell death after 24 hr (lethal effect L.E.), and as the concentration able to induce morphological alterations (cytotoxic effect C.E.) after 24 hr.

RESULTS

In Table 1 the drugs studied have been grouped by their activity in the two experimental situations:

In group A drugs are collected which were both active in the TVR test and cytotoxic *in vitro*. They are actinomycin-D, aminochlorambucil, BCNU, CCNU, 5-fluorouracil, melphalan, procarbazine, triethylenemelamine, vinblastine and VM 26. In group B are drugs for which the agreement between *in vitro* and *in vivo* results is based on their total inactivity. They are 6-aminochrysene in both its physical preparations, 6-mercaptopurine and methotrexate. In the next two groups are compounds having different activity in the two experimental situations. In group C the compound active *in vivo* but not cytotoxic per se is represented by cyclophosphamide. Drugs cytotoxic *in vitro* but inactive *in vivo* are grouped in D, they are: Ara-C, CB 1954, chlorambucil, diazouracil, emetine, hydroxyurea, azathioprine and mechlorethamine. To test the influence of the schedule of administration on the TVR test, several compounds have been evaluated *in vivo* by giving to the animals three subcutaneous injections at 4 hr intervals at a 10% lethal dose level. Table 2 compares the results obtained in this way with the previous results. By this schedule Ara-C, hydroxyurea and azathioprine are seen to be active also *in vivo* whereas emetine still is inactive. The activity of procarbazine remains unchanged, vinblastine shows partially increased activity upon fractionated administration and methotrexate and 6-mercaptopurine are found active in the TVR test despite their inactivity *in vitro*.

DISCUSSION

The interpretation of these results can be misleading if the limitations of the two experimental situations are not clearly set.

The *in vitro* effect has been measured over 24 hr of cell drug interaction in the absence of quantitatively relevant drug metabolism.

The morphologic evaluation performed by phase contrast microscopy is more sensitive in revealing early cytolysis produced by cytotoxic compounds than the growth inhibition induced by antimetabolites which may show delayed toxicity only. The *in vivo* evaluation of tumour volume reduction is affected by drug metabolism in the host and the acute LD_{10} given to the animals is likely to produce a high peak level of drug that will last for a length of time that may

Table 1. *C22 LR osteosarcoma sensitivity compared* in vitro *and* in vivo

Drugs	*In vitro* Lethal concentration (μg/ml)	*In vitro* Cytotoxic concentration (μg/ml)	*In vivo* LD_{10} (μg/g)	*In vivo* Tumour volume reduction factor
(A) *Drug cytotoxic* in vitro *and* in vivo				
Actinomycin D	0·1	0·1	0·6 i.p.	0·47+
Aminochlorambucil	0·1	0·01	20 s.c.	0·39+
BCNU	0·1	0·1	40 s.c.	0·20++
CCNU	1	1	100 s.c.	0·17++
5-Fluorouracil	> 100	100	350 s.c.	0·34+
Melphalan	1	1	32 s.c.	0·39+
Procarbazine*	> 100	1	750 s.c.	0·40+
Triethylenemelamine	1	1	2·5 s.c.	0·27+
Vinblastine	0·1	0·01	20 s.c.	0·20++
VM 26	0·1	< 0·1	150 s.c.	0·14++
(B) *Drugs inactive* in vitro *and* in vivo				
6-Aminochrysene suspension	> 100	> 100	100 i.p.	0·93
6-Aminochrysene micronized	> 100	> 100	50 i.p.	0·84
6-Mercaptopurine*	> 100	> 100	150 s.c.	0·70
Methotrexate*	> 100	> 100	150 s.c.	0·70
(C) *Drugs non-cytotoxic* in vitro *but active* in vivo				
Cyclophosphamide	> 100	> 100	200 sc	0·14++
(D) *Drugs cytotoxic* in vitro *but inactive* in vivo				
Ara-C*	0·1	0·1	1200 s.c.	0·70
CB 1954	10	10	100 s.c.	0·64
Chlorambucil	10	10	25 s.c.	0·59
Diazouracil	1	1	22 s.c.	0·58
Emetine	< 0·01	< 0·01	25 s.c.	0·67
Hydroxyurea	> 100	100	2400 s.c.	0·72
Azathioprine*	10	10	375 s.c.	0·70
Mechlorethamine	1	0·1	2 s.c.	0·50

*Also tested *in vivo* by fractionated administration. See text and Table 2.

Table 2

Drugs	*In vitro* Lethal concentration (μg/ml)	*In vitro* Cytotoxic concentration (μg/ml)	Fractionated dose LD_{10} (μg/g)	*In vivo* Tumour volume reduction Single dose	*In vivo* Tumour volume reduction Fractionated dose*	Group
Ara-C	0·1	0·1	1200 s.c.	0·70	0·40+	A
Emetine	< 0·01	< 0·01	25 s.c.	0·67	0·74	D
Hydroxyurea	> 100	100	2400 s.c.	0·72	0·27+	C
Azathioprine	10	10	375 s.c.	0·70	0·38+	A
6-Mercaptopurine	< 100	> 100	150 s.c.	0·70	0·43+	C
Methotrexate	> 100	> 100	150 s.c.	0·70	0·49+	C
Procarbazine	> 100	1	900 s.c.	0·40+	0·37+	A
Vinblastine	0·1	0·1	18 s.c.	0·20++	0·04++	A

*This total dose was given fractionated in three injections at 4 hr intervals.
The total quantity (three dose LD_{10}) was sometimes different from the single dose LD_{10} as the toxicity varied with the administration schedule.

be too short to permit a sufficient uptake of the compounds by the cells. The analysis of the results is made easier if these points are kept in mind. Among compounds grouped in A as cytotoxic *in vitro* and inactive *in vivo*, it must be noted that 5-fluorouracil appears to be non-lethal at a very high concentration despite evidence of cytotoxic alterations in the cell population. This can be a consequence of the presence of a small amount of thymidine in the non-dialyzed serum in the medium [6]. A similar low effectiveness can be noted for procarbazine. However, this drug is much more effective when judged on the basis of the CE. For both these compounds, the LE_{10} doses are so high that it seems possible that the highest concentration tested *in vitro* could be lower than the drug levels obtainable *in vivo*.

In group B the compounds ineffective *in vitro* and *in vivo* seem indeed to be effective only at drug levels which cannot be reached *in vivo*. 6-aminochrysene is known to be specific for breast cancer and to need metabolic transformation [7]. It might belong to group C if tested on breast cancer. For 6-mercaptopurine and methotrexate the inactivity *in vivo* is related only to the short contact between drug and cells *in vivo* after a single injection.

After fractionation of the dose, these two drugs show activity *in vivo*. In our experience in this and other tumour cultures, the *in vitro* test never recorded any activity of these two compounds. For 6-mercaptopurine, metabolic transformation occurs *in vivo* [8] but for methotrexate this does not seem likely since it was found active *in vitro* in other systems [9]. Also, in this case, the minimal amounts of thymidine which are present in the medium might be sufficient to prevent *in vitro* toxicity.

In group C only cyclophosphamide represents drugs that are inactive *in vitro* but can be activated *in vivo* after metabolic transformation. As seen before, 6-aminochrysene and 6-mercaptopurine may belong to this group when other tumour models are considered. These compounds never should be tested *in vitro* as such, but their metabolites should be used for studies *in vitro*.

Group D contains drugs which are effective *in vitro*, but ineffective *in vivo*. This is demonstrated most clearly for emetine, which is extremely effective *in vitro*. Its ineffectiveness *in vivo* may be due to the presence of an excess of binding sites in muscle and liver [10] which could be the cause of its low concentration in the serum and possibly in the tumour cells of mice. Blood levels after therapeutic doses for amebiasis in man remain only for a short time over 1 ppm indicating that most of the drug is rapidly removed from the circulation.

Among the other compounds, Ara-C, hydroxyurea and azathioprine, when given in fractioned doses, are seen to belong to group A, mechlorethamine possibly owes its irregular position in this group to its steep toxicity curve, which makes a fine adjustment of the LD_{10} dose difficult, and to the threshold value of its TVR. CB 1954, chlorambucil and diazouracil have not been tested in a fractionated schedule.

In conclusion, it will be clear that discrepancies between *in vivo* and *in vitro* effects can be a consequence of:

(*a*) absence *in vitro* of a metabolic transformation system (cyclophosphamide, 6-mercaptopurine, 6-aminochrysene);
(*b*) presence *in vitro* of competitors for antimetabolites (5-fluorouracil, methotrexate ?);
(*c*) too low tumour drug levels which can be reached by safe drug doses (emetine ?);
(*d*) short duration of effective drug levels after single doses *in vivo* (methotrexate, Ara-C, hydroxyurea, azathioprine);
(*e*) unidentified factors (CB 1954, chlorambucil, mechlorethamine and diazouracil).

By expanding studies of this type to other experimental tumours, it seems that a least for some widely used drugs, satisfactory *in vitro* techniques can be identified which may permit prediction of clinical usefulness in a tumour therapy schedule.

REFERENCES

1. L. Morasca, G. Balconi, F. De Nadai and E. Dolfini, Chemotherapeutic "fingerprints" of two experimental tumours *in vitro*. *Europ. J. Cancer* **8,** 429 (1972).
2. E. Dolfini, A. Martini, M. G. Donelli, L. Morasca and S. Garattini, Method for tissue culture evaluation of the cytotoxic activity of drugs active through the formation of metabolites. *Europ. J. Cancer* **9,** 375 (1973).
3. L. M. van Putten, Tumour reoxygenation during fractionated radiotherapy; studies with a transplantable mouse osteosarcoma. *Europ. J. Cancer* **4,** 173 (1968).

4. G. G. Rose, C. M. Pomerat, T. O. Shindler and J. B. A. Trunnel, A cellophane-strip technique for culturing tissue in multipurpose culture chambers. *J. biophys. biochem. Cytol.* **4,** 761 (1958).
5. G. Balconi, A. Bossi, M. G. Donelli, S. Filippeschi, G. Franchi, L. Morasca and S. Garattini, Chemotherapy of a spontaneous mammary carcinoma in mice: relation between *in vitro–in vivo* activity and blood and tumor concentrations of several antitumoral drugs. *Cancer Chemother. Rep.* **57,** 115 (1973).
6. H. Madoc-Jones and W. R. Bruce, Sensitivity of L-cells in exponential and stationary phase to 5-fluorouracil. *Nature (Lond.)* **215,** 302 (1967).
7. G. Franchi, L. Morasca and S. Garattini, Attività' antitumorale di alcuni derivati del 6-NH_2 crisene. *Tumori* **55,** 333 (1969).
8. M. G. Donelli, T. Colombo, A. Forgione and S. Garattini, Distribution of 6-mercaptopurine in tumor-bearing animals. *Pharmacology* **8,** 311 (1972).
9. W. M. Hryniuk and J. R. Bertino, Growth rate and cell kill. *Ann. N.Y. Acad. Sci.* **186,** 330 (1971).
10. A. I. Gimble, C. Davison and P. Smith, Studies on the toxicity, distribution and excretion of emetine. *J. Pharmacol. exp. Ther.* **94,** 431 (1948).

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Suppression of Transplanted Rat Sarcomata Mediated by Bacillus Calmette Guérin (BCG)*

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Abstract—*Two rat sarcomata which normally grow progressively on transplantation into syngeneic recipients failed to develop local tumours when injected in admixture with living Bacillus Calmette Guérin (BCG), while intralesional infiltration of clinically established neoplasms retarded tumour growth and occasionally induced complete regression. By contrast, the local development of a third neoplasm was temporarily inhibited, but the incidence of spontaneous metastases was markedly reduced.*

Optimal suppression required living BCG, although heat-inactivated preparations were also partially inhibitory. Sonicated BCG and soluble purified protein derivative (PPD) were without significant effect.

Intimate contact of tumour cells and mycobacteria was essential for growth inhibition. In vitro *tests minimized the likelihood that tumour inhibition was attributable to a direct cytocidal effect of BCG or its products. The effects thus appeared to be dependent on host responses to the bacterial–tumour cell inoculum. The capacity of BCG to suppress tumour growth bore no clear relationship to the innate immunogenicity of the neoplasms as determined by conventional transplantation tests. However, resistance to subsequent tumour challenge developed in rats where the outgrowth of antigenic tumour cells was prevented by prior admixture with BCG.*

The possible role of immune responses to bacterial and tumour antigens in BCG-mediated tumour suppression is discussed.

INTRODUCTION

THERE is now considerable evidence that clinical cutaneous neoplasms may undergo regression in the vicinity of delayed hypersensitivity reactions induced by vaccinia virus [1] chemical agents [2, 3] and the Calmette Guérin strain of *Mycobacterium bovis* (BCG) [4, 5].

Several experimental models of this phenomenon have been proposed, particularly in respect of the capacity of BCG to inhibit growth of different tumour types in a number of species [6–9]. The involvement of host responses which are primarily immunological in nature has motivated critical assessment of the ability of BCG to suppress the growth of rat tumours of different immunogenicity and contrasting biological behaviour, with the ultimate objective of delineating those components of the host response which contribute to successful BCG-mediated tumour growth inhibition. In this paper some conditions under which BCG has proved inhibitory for three rat sarcomas

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transplanted within the strain of origin are defined.

Part of this study made use of two tumours of presumptively weak immunogenicity. These neoplasms—one of which metastasized spontaneously—were selected deliberately since immunopotentiating agents would probably be most beneficial in the treatment of poorly immunogenic tumours.

MATERIAL AND METHODS

Rats. The animals used in this study were female rats of the AS strain, maintained in our colony by strict brother-sister mating and tested regularly for genetic uniformity by skin grafting.

Tumours. The rat sarcomata (Table 1) were induced chemically or by internal irradiation [10] and were routinely transplanted to young adult rats of the same sex as the original tumour-bearer. Each tumour was used between 20 and 30 generations of passage.

Tumours were dissociated into single cell suspensions by treatment with 0·25 % trypsin in Hanks balanced salt solution. After washing by centrifugation and resuspension in tissue culture medium, the cells were assessed for viability (usually >90 %) by trypan blue exclusion.

Bacillus Calmette Guérin (BCG). BCG was obtained from Glaxo Laboratories, Greenford, Middlesex, England, as freeze-dried, percutaneous vaccine, batch numbers P620 and P642. These contained 94 and 93 million viable units per ampoule respectively, which proportionally accounted for approximately 30 % of the vaccine. The reconstituted preparations thus comprised 70 % non-viable material. Prior to injection, the BCG was suspended in water to give 3 mg/ml. Ultrasonicated BCG was also provided by Glaxo Laboratories and heat-inactivated vaccine was prepared by heating the microorganisms for 1 hr at 65°C. Both preparations were checked for sterility by failure to establish cultures in media and at temperatures which supported the growth of organisms from the reconstituted, but otherwise untreated bacilliary suspensions viz. buffered Lowenstein-Jensen medium, Pyruvate egg medium and Kirchner's medium. The freeze-drying medium (dextran 8·3 % w/v; glucose 7·5 % w/v and Triton WR 1339 1/4000 v/v)was employed for controls in early tumour-inhibition tests but was subsequently substituted by tissue culture medium.

Assessment of tumour growth inhibition in vivo

Effect of BCG on outgrowth of subcutaneously inoculated tumour cells. Equal volumes of suspension containing tumour cells and reconstituted microorganisms were mixed immediately prior to injection so as to give the appropriate number of tumour and bacterial cells in 0·1 ml suspension for subcutaneous inoculation. The dependence of tumour growth inhibition on intimate contact of BCG with tumour cells was evaluated in experiments where they were injected at separate subcutaneous sites.

Effect of BCG on clinically established subcutaneous neoplasms. BCG ($1{\cdot}25 \times 10^6$ viable microorganisms; 400 μg reconstituted vaccine) in 0·1 ml. was infiltrated centrally into tumour nodules ranging from 6 to 9 mm dia.

The effect of BCG on the growth of admixed tumour cells and on clinically established neoplasms was estimated by twice weekly caliper measurements of two tumour diameters at right angles to each other, and recording final tumour incidences for the respective groups. In the case of experiments using the metastatic fibrosarcoma (P8), pulmonary metastases were enumerated and measured following perfusion of lungs with india ink and fixation in Fekete's solution [11].

Assay of tumour cell growth inhibition in vitro

These tests were carried out on tertiary monolayers derived from osteosarcoma CC5

Table 1. Summary of properties of rat sarcomata

Tumour	Site and histology of primary neoplasm	Oncogenic agent	Minimum inoculum for growth*	Maximum inoculum rejected by immune hosts†
CC5	femur/osteosarcoma	N-hydroxy-2-acetyl amino fluorene (cupric chelate)	1×10^5	2×10^6
P7	tibia/osteosarcoma	Radiophosphorus (P^{32})	5×10^4	1×10^5
P8	femur/fibrosarcoma	Radiophosphorus (P^{32})	1×10^4	1×10^4

*Number of cells required to produce tumour takes, on subcutaneous inoculation, in ≥ 80% untreated recipients.
†Number of cells rejected by ≥ 80% specifically preimmunized recipients.

and grown in Eagles minimal essential medium (MEM) supplemented with 20 % foetal bovine serum and antibiotics. The neoplastic nature of the cells was verified by their ability to grow progressively on reinoculation into syngeneic recipients.

Tumour cells were harvested from monolayers by trypsinization and bacterial–tumour cell suspensions in supplemented Eagles MEM (but without antibiotics) were prepared for plating by 100-fold dilution of mixtures from which the *in vivo* outgrowth of osteosarcoma cells was optimally suppressed. Microplate wells (Falcon No. 3034) were inoculated with 10 μl of this suspension such that each test well received 100 tumour cells in admixture with 125 or 250 viable BCG organisms, and control wells received tumour cells only. Incubation was for 48 hr at 37°C in an atmosphere of 5 % CO_2 in air, after which each plate was stained with crystal violet and the number of spreading tumour cells enumerated.

Induction of tumour immunity in vivo

To assess the capacity of admixed tumour and bacterial cells to elicit tumour immunity, groups of rats were challenged contralaterally with graded viable tumour inocula simultaneously and at various time intervals after treatment. The tumour incidence in the BCG: tumour cell treated rats was compared with that in normal, untreated recipients. Only osteosarcoma CC5, the one tumour of unequivocal immunogenicity as determined by transplantation tests, was used for this purpose.

RESULTS

Tumour growth inhibition of two transplanted osteosarcomas at the site of infection with living BCG

The capacity of BCG to inhibit the growth of the strongly antigenic chemically-induced osteosarcoma CC5, and the weakly antigenic radiation-induced osteosarcoma, P7 was evaluated in two experimental situations: First, the prevention of outgrowth into clinically detectable neoplasms of tumour cells which had been admixed with living BCG immediately prior to inoculation; and second, the inhibition of clinically established tumours by intralesional infiltration with vaccine containing viable microorganisms.

To test the ability of BCG to suppress the

Table 2. Inhibition of growth of transplanted rat osteosarcoma CC5 at the site of infection with living BCG

	BCG			Fraction of rats developing tumours			
Tumour cells grafted	No. of viable organisms ($\times 10^{-6}$)	Mode of administration	Time (days)	BCG-treated rats	Latent* period	Untreated controls	Latent* period
1×10^6	2·50	admixed; s.c.	0	0/5	—	5/5‡	11
1×10^6	1·25	contralateral; s.c.	−23†	5/5	11	5/5	11
1×10^6	1·25	admixed; s.c.	0	1/14	11	15/15	11
1×10^6	1·25	contralateral; s.c.	0	10/10	11	5/5	11
1×10^6	1·25	admixed; s.c.	0	0/4	—		
1×10^6	0·63	admixed; s.c.	0	3/4	13		
1×10^6	0·32	admixed; s.c.	0	2/4	13		
1×10^6	0·16	admixed; s.c.	0	1/4	13	16/16	13
1×10^6	0·063	admixed; s.c.	0	1/4	13		
1×10^6	0·031	admixed; s.c.	0	4/4	13		
1×10^6	0·016	admixed; s.c.	0	3/3	13		
2×10^6	1·25	admixed; s.c.	0	0/5	—		
5×10^6	1·25	admixed; s.c.	0	1/5	12	5/5§	10
1×10^7	1·25	admixed; s.c.	0	2/5	11		
1×10^6	1·25	intralesional	+12[1]	9/10	—	10/10	12
5×10^5	1·25	intralesional	+28[2]	5/5	—	5/5	28
1×10^5	1·25	intralesional	+28[3]	4/5	—	5/5	28

*Time in days to first palpable tumour.
†Rats received 3 inoculations of BCG ($1{\cdot}25 \times 10^6$ viable organisms) at 7 day intervals; final injection 23 days prior to tumour cell challenge.
‡Tumour cells inoculated in BCG freeze-drying medium; in all other tests tissue culture medium was used.
§Controls received minimum inoculation (2×10^6 cells s.c.).
[1]Median tumour diameter of group, 7·4 mm.
[2]Median tumour diameter of group, 8.3 mm.
[3]Median tumour diameter of group, 7·0 mm.

outgrowth of transplanted tumour cells, a standard inoculum of osteosarcoma cells (usually 1×10^6) was grafted subcutaneously with variable numbers of viable (and non-viable) microorganisms in syngeneic recipients and the emergent tumours enumerated in BCG-treated rats and the corresponding controls receiving tumour cells only administered in serum-free tissue culture fluid or BCG freeze-drying medium.

Doses of BCG down to $6{\cdot}3 \times 10^4$ viable organisms (equivalent to 20 μg BCG) per million tumour cells were partially inhibitory for osteosarcoma CC5, but suppression of outgrowth of this standard inoculum was most effective and reproducible at higher doses ($1{\cdot}25 \times 10^6$ viable microorganisms; equivalent to 400 μg BCG) (Table 2). Thus, only 1/33 (3 %) rats receiving 10^6 CC5 osteosarcoma cells admixed with $1{\cdot}25 \times 10^6$ viable BCG organisms developed a tumour, whereas the outgrowth of 10^6 tumour cells alone in 41 rats was 100 %. Although tests with smaller numbers of animals indicated that the outgrowth of tumours in rats receiving between two and ten times the number of tumour cells could be restrained by the same number of BCG organisms, admixture of 10^6 osteosarcoma cells with $1{\cdot}25 \times 10^6$ viable microorganisms was considered optimal for effective suppression of outgrowth of this neoplasm. In the minority of rats which developed tumours from inocula admixed with sub-optimal doses of BCG, the time of appearance of the palpable neoplasms coincided closely with those in controls and their growth rate was also the same.

Essentially similar results were obtained with the weakly antigenic osteosarcoma, P7, although apparently fewer BCG viable organisms ($3{\cdot}2 \times 10^5$) were effective for the consistent suppression of growth of one million cells of this neoplasm (Table 3). In two experiments with tumours CC5 and P7 where rats received a simultaneous contralateral inoculation of BCG microorganisms, there was no difference in tumour incidence or in the rate of outgrowth, compared with controls; nor was there any inhibitory effect in rats which received a course of BCG immunization prior to challenge with CC5 sarcoma cells. The necessity for intimate contact of the BCG with the tumour cells for effective suppression of growth was thereby emphasized.

By contrast with the marked inhibition of tumour growth obtained when tumour cells were injected in admixture with BCG, infiltration of clinically established tumour nodules of both CC5 and P7 by intralesional injection of living bacteria was largely ineffective. Thus only 1/10 BCG-infiltrated CC5 tumours originating from the inoculation of 1×10^6 cells 12 days previously and 1/10 tumours originating from smaller inocula (5×10^5 and 1×10^5 cells) over a longer latency period (28 days), underwent complete regression at this BCG dosage (Table 2), although the growth rate of individual neoplasms receiving intralesional BCG was frequently temporarily retarded. Whether inoculation of BCG at an earlier stage of tumour development, or infiltration of larger numbers of viable microorganisms would have effectively altered the clinical course of these established tumours was not investigated.

Effect of BCG on growth of a spontaneously metastasizing fibrosarcoma (P8)

Fibrosarcoma (P8) cells inoculated subcutaneously into syngeneic recipients develop

Table 3. Inhibition of growth of transplanted rat osteosarcoma P7 at the site of infection with living BCG

	BCG			Fraction of rats developing tumours			
Tumour cells grafted	No. of viable organisms ($\times 10^{-6}$)	Mode of administration	Time (Days)	BCG-treated rats	Latent period*	Untreated controls	Latent period*
1×10^6	2·50	admixed; s.c.	0	0/5	—		
1×10^6	1·25	admixed; s.c.	0	0/10	—		
1×10^6	1·25	contralateral; s.c.	0	5/5	10	10/10	10
1×10^6	0·32	admixed; s.c.	0	0/5	—		
1×10^6	1·25	intralesional	+13‡	5/5	—		
2×10^6	1·25	admixed; s.c.	0	0/6	—	5/5†	10
6×10^6	1·25	admixed; s.c.	0	1/6	6	5/5	6
3×10^7	1·25	admixed; s.c.	0	5/5	5	5/5	5

*Time in days to first palpable tumour.
†Tumour cells inoculated in BCG freeze-drying medium; in all other tests, tissue culture medium was used.
‡Median tumour diameter of group, 6·5 mm.

Table 4. Prevention of metastases of fibrosarcoma cells (P8) inoculated subcutaneously in admixture with living BCG

Group	No. of rats treated	No. of tumour cells	BCG: No. of viable organisms ($\times 10^{-6}$)	BCG: Mode of administration	BCG: Time (days)	Mean diameter ± SD s.c. tumour	*P*	Fraction of rats with lung nodules*	Mean no. ± SD lung nodules	*P*	Mean diameter ± SD of lung nodules	*P*	No. of rats with other metastases
1A	5	10^4	1·25	admixed; s.c.	0	25·8 ± 3·6	< 0·008	0/5	0	< 0·008	0	< 0·008	none
2A	5	10^4	1·25	contralateral; s.c.	0	46·0 ± 4·6	N.S.	5/5	9·0 ± 4·2	N.S.	4·8 ± 1·3	< 0·02†	1 (liver) 2 (lymph nodes)
3A	5	10^4	1·25	intralesional	+22	45·0 ± 2·5	N.S.	5/5	10·0 ± 6·3	N.S.	4·0 ± 2·0	N.S.	1 (liver) 1 (spleen) 1 (lymph nodes)
4A	5	10^4	0	none (controls)	0	48·2 ± 4·2	—	5/5	8·2 ± 4.6	—	1·9 ± 0·9	—	2 (liver) 2 (lymph nodes)
1B	5	10^5	1·25	admixed; s.c.	0	32·3 ± 7·7	< 0·02	2/5	0§	< 0·008	not determined		none
2B	5	10^5	1·25	contralateral; s.c.	0	51·8 ± 5·2	N.S.	5/5	11§	N.S.	not determined		2 (liver) 2 (stomach) 1 (spleen)
3B	4	10^5	1·25	intralesional	+22	41·6 ± 6·0	N.S.	4/4	5·5§	N.S.	not determined		2 (liver) 2 (spleen) 1 (stomach)
4B	5	10^5	0	none (controls)	0	49·1 ± 7·4	—	5/5	9§	—	not determined		1 (liver)

N.S. = not significant;
*Experiments terminated on Day 86;
†significant enhancement;
§median values quoted since lungs of some animals almost entirely replaced by metastatic tumour.
All statistical analyses by Mann–Whitney *U*-test.

into local neoplasms from small inocula of tumour cells and invariably develop metastatic deposits in distant organs. Pulmonary tumours develop in 100 % of rats receiving tumour transplants but in untreated hosts, metastatic deposits may also be found in other organs, particularly the liver and lymphoid tissues.

In two experiments designed to assess the effect of BCG on local tumour outgrowth and on the development of metastases, small numbers of fibrosarcoma cells (10^4 and 10^5 cells) were admixed prior to s.c. inoculation with $1{\cdot}25 \times 10^6$ viable BCG organisms. Each experiment comprised four groups of rats of which the groups 1A and 1B received tumour cells admixed with BCG inoculated subcutaneously (Table 4); groups 2A and 2B received comparable numbers of tumour cells on one flank and the equivalent number of BCG organisms contralaterally; groups 3A and 3B received $1{\cdot}25 \times 10^6$ organisms BCG intralesionally when the tumours became clinically detectable and groups 4A and 4B received tumour cells alone. For each tumour cell inoculum, the time of appearance and rate of development of tumours in groups 2, 3 and 4 were identical and the mean tumour diameter profiles with respect to time, superimposable (as shown in Fig. 1 for the 10^4 cell inoculum). However, there was a significant delay in the clinical appearance of tumours in rats in groups 1A and 1B compared with the other groups, where rats received P8 cells admixed with $1{\cdot}25 \times 10^6$ viable BCG organisms, which was particularly apparent in the group (1A) receiving the lower (10^4) tumour cell inoculum (Fig. 1). However, by contrast with CC5 and P7, complete suppression of subcutaneous tumour outgrowth was not observed. Both experiments were terminated on day 86, when all rats were examined for distant tumour deposits *post mortem*. At this time, the mean diameter of s.c. tumours in rats which received contralateral or intralesional BCG (groups 2 and 3) did not differ significantly from controls (group 4), whereas the mean tumour diameter of neoplasms in rats which received tumour cells and BCG admixed was significantly less (groups 1A and 1B) in each experiment (Table 4).

The most marked differences between groups receiving tumour cells admixed with BCG and the other treatment schedules was in the number and size of metastases to distant organs, particularly the lungs. In Experiment A, 100 % of rats which received tumour cells alone, contralateral or intralesional BCG developed pulmonary nodules whereas none were present in rats which had received the mixed bacterial-tumour cell inoculum. In the group receiving BCG contralaterally to the site of tumour cell injection, (Group 2A), there was significant enhancement in size of the pulmonary tumour deposits, compared with controls (Group 4A). In Experiment B, although pulmonary deposits developed in 2/5 rats receiving the higher s.c. inoculum (10^5) of P8 fibrosarcoma cells admixed with BCG, the number and size of the lung metastases in this group were significantly less than in the other treatment groups, where not infrequently whole lungs were replaced by tumour tissue, thus

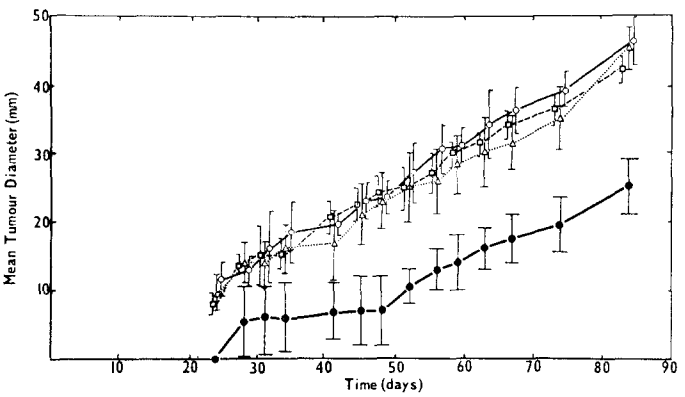


Fig. 1. Partial inhibition of subcutaneous growth of fibrosarcoma (P8) cells (10^4) following admixture with living BCG ($1{\cdot}25 \times 10^6$ organisms).

Tumour cells admixed with BCG (Group 1A), ●——●; BCG administered contralateral to tumour cells (Group 2A), △.....△; BCG infiltrated intralesionally to clinically palpable tumour (Group 3A), □- - -□; and tumour cells only (Group 4A), ○——○. Experimental groups correspond with those in the text and Table 4.

permitting analysis of the number of pulmonary lesions in rats of this series, by rank only. Histological examination of serial sections of the lungs of those rats in groups 1A and 1B which were macroscopically tumour free failed to reveal any evidence of micrometastases [12].

Effect of living BCG on the viability of cultured osteosarcoma cells (CC5) in vitro

While the outgrowth of tumour from small inocula ($10^4/10^5$ cells) of P8 fibrosarcoma admixed with living BCG rendered unlikely the possibility that BCG may be directly cytocidal for tumour cells, this was confirmed by tests on the effect of the living bacteria on cultured osteosarcoma cells (CC5) against which BCG was inhibitory *in vivo*. The results of three independent tests (Table 5) showed

Table 5. *Effect of living BCG on viability of CC5 tumour cells* in vitro

Mixed cell inoculum plated		No. of cells after 48 hr (No. of wells counted)		
No. of tumour cells	No. of viable BCG organisms ($\times 10^{-2}$)	Control	Test	P*
10^2	1·25	207·1 ± 37·1 (12)	205·8 ± 39·6 (20)	N.S.
10^2	1·25	141·3 ± 29·6 (12)	149·1 ± 15·7 (16)	N.S.
10^2	2·50	155·4 ± 17·1 (12)	151·8 ± 16·4 (28)	N.S.

*By Students *t*-test.
N.S. not significant.

that the numbers of tumour cells grown in the presence and absence of BCG did not differ significantly.

Requirement of living BCG for optimal suppression of tumour growth

Since the reconstituted BCG vaccines used in this study contained more dead than living bacilli it was important to determine the extent to which viability was a factor in the effective inhibition of transplanted osteosarcoma CC5. Tumour cells were therefore variously admixed with inactive BCG microorganisms, the approximate untreated equivalent of which optimally suppressed the outgrowth of a standard inoculum of 10^6 osteosarcoma cells. Three test groups comprised rats which received tumour cells which had been admixed prior to inoculation with (i) heat-inactivated BCG (ii) ultrasonicated BCG and (iii) PPD-purified protein derivative of BCG. The rate of growth and final incidence of emergent tumours in each group was compared with that in rats receiving tumour cells alone on the one hand, and tumour admixed with vaccine containing living bacteria ($1{\cdot}25 \times 10^6$ organisms) on the other.

Purified protein derivative (PPD 200 μg) and the sonicated BCG preparations were least effective, tumours appearing in the majority of rats so treated and with comparable latency periods to those in controls (Fig. 2). However,

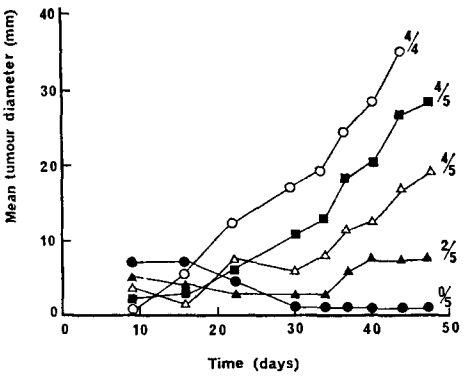


Fig. 2. *Comparison of the* in vivo *outgrowth of CC5 osteosarcoma (10^6 cells) from admixtures with untreated and inactivated BCG vaccine and semi-purified protein derivative (PPD).*

●——● *Living BCG ($1{\cdot}25 \times 10^6$ organisms).* ▲——▲ *Heat-killed BCG.* △——△ *Sonicated BCG.* ■ ■ *Soluble purified protein derivative (PPD).* ○——○ *Controls; no BCG.*

significant inhibition of growth of tumour cells admixed with heat-killed bacteria was observed, although the degree of suppression assessed by the final tumour incidence in this group (40 %) was less than in rats which received tumour cells admixed with vaccine containing viable organisms where growth inhibition was 100 %.

Development of tumour immunity

Tests were undertaken with the strongly antigenic osteosarcoma CC5 to assess the degree of tumour immunity induced by the bacterial–tumour cell mixtures.

Evidence of tumour resistance was obtained in rats which received tumour challenges of 2×10^5 cells contralaterally and at the same time as the immunizing mixed tumour–BCG inoculum, only 2/7 rats developing tumours compared with 5/5 controls (Table 6). The degree of immunity was relatively weak, however, since rats were susceptible to an increased challenge dose of 10^6 cells, although the rate of growth of tumours in treated rats was marginally retarded compared with

Table 6. Development of anti-tumour immunity following treatment with CC5 osteosarcoma cells admixed with living BCG ($1{\cdot}25 \times 10^6$ organisms)

Tumour challenge*		Fraction of rats with tumours	
No. of CC5 cells	Days after treatment	BCG: tumour cell-treated	Untreated controls
2×10^5	0	2/7	5/5
1×10^6	0	10/10	8/8
2×10^5	10	0/7	4/4
1×10^6	10	2/5	5/5
1×10^6	20	5/5	4/4
1×10^6	30	3/4	3/3
1×10^5	43	0/6	5/5
1×10^5	58	1/3	5/5†
5×10^5		4/4	
1×10^6		2/3	

*Tumour challenge inoculated contralaterally to tumour–bacterial cell mixtures.

†Controls received minimum inoculum (1×10^5 cells s.c.).

controls. When rats were challenged with CC5 tumour cells 10 days after immunization with the mixed tumour–bacterial cell inoculum, the induced resistance appeared to be more pronounced, 0/7 and 2/5 rats developing neoplasms in the groups which received 2×10^5 and 1×10^6 cells respectively, compared to 100% outgrowth in each of the control groups. In further tests in which the contralateral injection of challenge inocula (10^6 cells) was delayed by 20 and 30 days, the majority of recipients succumbed to tumour challenge. However, reduction in the level of the challenge inoculum by one logarithmic unit (10^5 cells) revealed a group of rats which were tumour-resistant 43 days after treatment with the tumour–bacterial cell mixture, while in other smaller groups challenged with 10^5–10^6 cells at 58 days, resistance was noticeably less consistent.

DISCUSSION

The objective of this study was definition of the conditions under which BCG was inhibitory for a group of experimentally-induced rat neoplasms of connective tissue derivation selected for study on account of their differences in biological behaviour and antigenic status. The development of tumours which normally grow progressively when transplanted subcutaneously into syngeneic recipients was markedly inhibited when injected in admixture with the Glaxo strain of Bacillus Calmette Guérin (BCG). Complete suppression of tumour outgrowth appeared to some extent to be a BCG dose-dependent phenomenon, the threshold inoculum required for inhibition of 10^6 tumour cells being of the order of 10^5 viable microorganisms. However, this observation must be interpreted with the awareness that the BCG reconstituted vaccines used in this study contained a large proportion of dead bacilli. While optimal tumour suppression required admixture with living microorganisms, heat-killed bacteria were not without effect. Physical disruption, on the other hand, markedly reduced the efficacy of the bacteria to inhibit tumour growth, while soluble semipurified mycobacterial antigen (PPD) was completely inactive in this system. These findings indicate that crude BCG vaccine contains antigenic constituents absent from the purified product, PPD, and that the capacity to inhibit tumour cell outgrowth is related to the manner of presentation of the bacterial cell antigens. Zbar *et al.* [13] have demonstrated that the tumour-suppressive property of the tubercle bacillus is associated with the cell wall. In their study, the immunological responsiveness of the host was found to be critically dependent upon the mode of presentation of the cell wall antigens. Thus, cell walls alone were ineffective while attachment to oil droplets restored tumour-suppressive capacity.

For osteosarcomas P7 and CC5 at least 50 times the threshold inoculum for progressive growth in >80% of recipients was inhibited by BCG. The effect could, however, be overridden at higher tumour–bacterial cell ratios. By contrast, the outgrowth of the threshold inoculum of metastatic fibrosarcoma P8 at the site of injection with BCG was only partially suppressed. However, marked reduction in the number and size of spontaneous pulmonary metastases was a striking feature of concomitant BCG infection at the site of subcutaneous inoculation of this tumour. Moreover, the inhibitory effect was not limited to pulmonary metastases since dissemination to organs other than the lungs was not infrequently encountered among rats in the various groups.

By contrast with the pronounced inhibitory effect of BCG on admixed tumour cells, complete regression of clinically established tumours by intralesional infiltration occurred rarely, although transient arrest of tumour growth was generally observed. Several limiting factors may be implicated in this experimental situation, of which the immunogenicity of the BCG is probably the most important. A strain of poor immunogenicity—attributed to the Glaxo product used in these studies [14]—would be

unlikely to evoke a sustained and destructive reaction at sites of implantation.

Intimate contact of BCG organisms with tumour cells was an essential prerequisite for inhibition of local tumour outgrowth as specified in other studies [7, 8]. Furthermore, in the case of the metastatic tumour, P8, the number of pulmonary deposits was reduced only when the subcutaneous inoculum of tumour cells contained admixed BCG. Contralateral administration of BCG before or simultaneously with the graft of tumour cells neither augmented host resistance to subcutaneous tumour challenge, nor prevented metastatic spread. Since co-cultivation *in vitro* of BCG and neoplastic cells produced no detectable tumour cytolysis, the suppression of tumour outgrowth *in vivo* could not be attributed to a direct cytotoxic effect of BCG or its products on the transplanted cells. Inhibition was thus dependent on host responses to the bacterial–tumour cell inoculum. Susceptibility to the inhibitory effects of BCG bore no simple relationship to the immunogenicity of the tumours as determined by customary transplantation tests viz. implantation and subsequent excision of viable tumour and immunization with irradiated tumour cells. By these techniques, one of the neoplasms (CC5) has proved to be consistently immunogenic over many generations of passage, whereas conclusive evidence for the antigenicity of sarcomas P7 and P8 has not been elicited [10]. However, injection of BCG in admixture with antigenic tumour cells generated a degree of tumour immunity. These findings, which are consistent with those of Baldwin and Pimm [8], indicate that the destruction of the tumour cells and hence the antigenic stimulus was not achieved so rapidly as to prevent the development of a measurable, albeit transient, level of tumour resistance. The importance of this component of the host immune response relative to that directed against the antigens associated with the mycobacteria has yet to be elucidated. Conceivably, as postulated by Bartlett *et al.* [7] the tumour cells may, in part, be destroyed non-specifically by virtue of their presence in the milieu of a host reaction to BCG which is predominantly immunological in nature (the so called "innocent bystander" effect).

The immunological implication of the requirement for contact between BCG and tumour cells would appear to be impingement on the same drainage lymph node. Studies with heterologous antigens such as sheep red blood cells in mice have shown that the modulating effect of living BCG on a superimposed antigenic stimulus, initially exercised within the regional lymphoid tissue, is exerted centrally and extends to both humoral and cellular responses [15]. More importantly, the induction of systemic immunity to a murine mastocytoma (P815) which had no detectable immunogenicity by conventional tests, was accomplished when irradiated tumour cells were inoculated into sites prepared by prior injection of BCG [16, 17]. Possibly the prevention of tumour dissemination following subcutaneous inoculation of BCG in admixture with spontaneously metastasizing fibrosarcoma cells may be attributable to modulation of the response to a tumour-specific antigen, indetectable by other transplantation procedures.

An alternative, but not mutually exclusive interpretation would be that since contact of BCG with tumour cells is apparently necessary for inhibition, BCG becomes systemically distributed in a manner comparable to that following intravenous or intradermal injection of the microorganisms in mice [18]. In several other studies, the anatomical site of tumour growth inhibition has frequently coincided with that of BCG infection, following systemic administration [19–22]. However, if this were the only mechanism in this instance, inhibition of tumour metastases in rats which received BCG contralateral to the site of tumour injection would have been expected, but this was manifestly not the case.

To what extent the anti-tumour effects of BCG may be attributed to potentiation of the host's immune response against the tumour, to systemic activation of the reticulo-endothelial system and to incidental destruction of tumour cells within the local granulomatous reaction aroused by BCG itself, or to a combination of two or more of these factors, remains to be elucidated. Consideration should be given also to a possible contribution by indirect factors of a non-immunological nature such as might arise, for instance, from BCG-induced changes in tissue stroma and vasculature.

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REFERENCES

1. I. Hunter-Craig, K. A. Newton, G. Westburg and B. W. Lacey, Use of vaccinia virus in the treatment of malignant melanoma. *Brit. med. J.* **2,** 512 (1970).
2. E. Klein, Hypersensitivity reactions at tumor sites. *Cancer Res.* **29,** 2351 (1969).
3. J. Stjernsward and A. Levin, Delayed hypersensitivity-induced regression of human neoplasms. *Cancer* **28,** 628 (1971).
4. D. L. Morton, Immunological studies with human neoplasms. *J. reticulo-endoth. Soc.* **10,** 137 (1971).
5. J. U. Gutterman, G. Mavligit, C. McBride, E. J. Frei, III, E. J. Freidrich and E. M. Hersh, Active immunotherapy with BCG for recurrent malignant melanoma. *Lancet* **i,** 1208 (1973).
6. B. Zbar, I. D. Bernstein and H. J. Rapp, Suppression of tumor growth at the site of infection with living Bacillus Calmette-Guérin. *J. nat. Cancer Inst.* **46,** 831 (1971).
7. G. L. Bartlett, B. Zbar and H. J. Rapp, Suppression of murine tumor growth by immune reaction to the Bacillus Calmette Guérin strain of mycobacterium bovis. *J. nat. Cancer Inst.* **48,** 245 (1972).
8. R. W. Baldwin and M. V. Pimm, Influence of BCG infection on growth of 3-methylcholanthrene-induced rat sarcomas. *Rev. Europ. Etudes clin. Biol.* **16,** 875 (1971).
9. B. Zbar, I. D. Bernstein, G. L. Bartlett, M. G. Hanna Jr. and H. J. Rapp, Immunotherapy of cancer: regression of intradermal tumors and prevention of growth of lymph node metastases after intralesional injection of living *Mycobacterium bovis*. *J. nat. Cancer Inst.* **49,** 119 (1972).
10. M. Moore, Tumour-associated antigens of experimentally-induced osteosarcomata. In *Bone: Certain Aspects of Neoplasia. Proc. of the 24th Symposium of the Colston Research Society*, (Edited by C. H. G. Price and F. G. M. Ross.) p. 411 Butterworths, (London) (1973).
11. H. Wexler, Accurate identification of experimental pulmonary metastases. *J. nat. Cancer Inst.* **36,** 641 (1966).
12. C. H. G. Price, Personal communication.
13. B. Zbar, H. J. Rapp and E. E. Ribi, Tumour suppression by cell walls of *Mycobacterium bovis* attached to oil droplets. *J. nat. Cancer Inst.* **48,** 831 (1972).
14. G. B. Mackaness, D. J. Auclair and P. H. Lagrange, Immunopoteniation with BCG. I. Immune response to different strains and preparations. *J. nat. Cancer Inst.* **51,** 1655 (1973).
15. T. E. Miller, G. B. Mackaness and P. H. Lagrange, Immunopotentiation with BCG. II. Modulation of the response to sheep red blood cells. *J. nat. Cancer Inst.* **51,** 1669 (1973).
16. E. Hawrylko and G. B. Mackaness, Immunopotentiation with BCG. III. Modulation of the response to a tumor-specific antigen. *J. nat. Cancer Inst.* **51,** 1677 (1973).
17. E. Hawrylko and G. B. Mackaness, Immunopotentiation with BCG. IV. Factors affecting the magnitude of an antitumor response. *J. nat. Cancer Inst.* **51,** 1683 (1973).
18. B. Zbar, Tumor regression mediated by *Mycobacterium bovis* (Strain BCG). *Nat. Cancer Inst. Monogr.* **35,** 341 (1972).
19. R. W. Baldwin and M. V. Pimm, BCG immunotherapy of pulmonary growths from intravenously transferred rat tumour cells. *Brit. J. Cancer* **27,** 48 (1973).
20. C. L. Larson, R. N. Ushijima, M. J. Florey, R. E. Baker and M. B. Baker, Effect of BCG on Friend disease in mice. *Nature (New Biol.)* **229,** 243 (1971).
21. G. Mathé, P. Pouillart and F. Lapeyraque, Active immunotherapy of L1210 leukaemia applied after the graft of tumour cells. *Brit. J. Cancer* **23,** 814 (1969).
22. R. W. Baldwin and M. V. Pimm, BCG immunotherapy of local subcutaneous growths and post-surgical pulmonary metastases of a transplanted rat epithelioma of spontaneous origin. *Int. J. Cancer* **12,** 420 (1973).

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Activity of Some Peptide Hydrolases in Muscle from Tumour-bearing Rats*

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Abstract—*To study the mechanism of muscle wasting in malignancy, rats were implanted with Yoshida sarcoma and the activity of a number of enzymes concerned in protein breakdown was measured in the extensor digitorum longus and soleus muscles. After* 20 *days of tumour growth, the body weights (excluding tumour) were approximately* 20% *less than controls. The extensor digitorum longus weighed, on average* 32% *less, but the soleus did not differ significantly. The activities per mg muscle protein in the extensor digitorum longus of acid proteinase, alkaline proteinase, arylamidase and cathepsin C were increased, whilst that of cathepsin B1 decreased; cathepsin A, autolytic activity and trypsin inhibitory capacity were unchanged. None of these activities was altered in the soleus. Total activities of most of the enzymes in the extensor digitorum longus were decreased, and none was increased. In the soleus there was an increase,* ($P < 0{\cdot}05$) *in the total leucine aminopeptidase.*

INTRODUCTION

AN ASSOCIATION of cachexia with malignant disease in humans is well-known and is a common cause of death in such patients [1]. In rats with growing tumours, there is widespread wasting of tissues, with muscle being particularly affected, as investigated in detail by Sherman *et al.* [2]. The conditions arising from the presence of the tumour, which are responsible for the loss of muscle protein have not been elucidated. On account of the large dietary demands of the tumour, together with possible interference with appetite or absorption of food in the host, these conditions may be identical with those responsible for protein loss in starvation. Alternatively, tumour-specific factors may be involved. In either event the possibility of mediation through hypersecretion of corticosteroids, having a protein catabolic action, must be considered. Also, that any effect on muscle is brought about, at least partly, by changes induced in the nervous system, cannot be ruled out.

Proteins of muscle, like those of other tissues, undergo continuous turnover. The average half-life of muscle proteins was found to be only about 6 days when errors due to reutilization of labelled amino acids were minimized [3]. Hence, a net loss of muscle protein could result either from diminished synthesis or increased catabolism or both. In isotope turnover studies, Goldberg [4] obtained evidence that muscle wasting following denervation or cortisone administration is associated with both an increased rate of breakdown and decreased synthesis of muscle protein. Such a complete study has not, however, been made with tumour-bearing animals, but Norberg and Greenberg [5], and more recently Clark and Goodlad [6] showed that there is a decreased rate of incorporation of injected labelled amino acid into muscle protein in such animals. Goodlad and Clark [7] have subsequently found a decreased ability of polyribosomes isolated from muscle of tumour-bearing rats to incorpo-

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rate [^{3}H]-lysine into protein, thus pointing to an impairment of the machinery of protein synthesis.

Little is known of the mechanism of protein breakdown in muscle. A number of peptide hydrolases have been identified in the tissue but are, in most cases, ill-defined and their relative contribution to protein breakdown in normal and pathological states is not clear. Benz and Lehmann [8] observed an increase (up to 3-fold) in proteinase activity, measured at pH 5·0, per unit wt of muscle, in homogenates of muscle from rats bearing Walker-256 carcinomas. Babson [9], however, found only slight increases in proteinase activity, measured at pH 3·5 and 7·5, in such animals. A significant increase in activity of acid proteinase (per unit of muscle protein) in rectus muscle from patients with malignant tumours has been reported recently [10]. Others, [11, 12] have reported increased acid proteinase in tissues, other than muscle, from tumour-bearing animals. An elevation in the activity of enzymes hydrolyzing leucine-β-naphthylamide and benzoyl-arginine-β-naphthylamide in liver, kidney and spleen of rats carrying human ovarian tumours has also been observed [13].

Much further work is required to ascertain the importance of peptide hydrolase activities in the loss of muscle protein in malignant states, both in general terms and as regards the relative importance of individual enzymes. It is even likely that some of these enzymes in muscle have yet to be identified and characterized. In the present work, the activity of a number of peptide hydrolases has been measured in muscle from rats carrying implanted tumours. In view of the fact that different types of muscle are known to respond differently to conditions which induce wasting, these studies were carried out with both soleus and extensor digitorum longus, which are, respectively, red and predominantly white muscle.

METHODS

Treatment of animals

Wistar rats of both sexes, weighing about 200 g, were used. Littermates of the same sex were paired on the basis of initial body wt, and one of the pair was injected with a tumour homogenate while the other was used as a control.

The tumour used was the solid Yoshida sarcoma. Tumours for inoculation (weighing about 15 g) were obtained from rats injected with tumour 8–10 days before sacrifice.

Littermate rats were anaesthetized with Nembutal (0·1 ml of a 1 in 5 dilution of Nembutal veterinary, Abbott Laboratories/50 g), and one rat of each pair was injected with 0·1 ml (approx. 100 mg) of finely chopped viable tumour tissue, so that the material passed into the superficial aspect of the biceps femoris muscle, while the other (control) rat was given a sham injection. The animals were subsequently fed *ad lib* with Thomson cube, Diet No. 1 (North Eastern Agricultural Co-operative Ltd.) The weight of each rat and the food consumption were recorded daily.

At various times after implantation of the tumour (see Tables) the animals were killed, and the extensor digitorum longus and soleus muscles removed from the right leg and weighed (minus tendon) as quickly as possible. They were chopped finely and homogenized in 1 ml of ice-cold 0·04M Tris–HCl (pH 7·6, measured at room temperature) per 100 mg of muscle. Homogenizing for two 30-sec periods with a loose-fitting Potter–Elvehjem type of glass homogenizer was sufficient to break up the fibres completely. The preparation was cooled briefly in ice after the first 30-sec period and was stored in ice after homogenizing. It was diluted with an appropriate volume of water for each assay.

Assay procedures

Acid proteinase activity in the homogenates was measured by incubating for 2 hr in 0·3 ml of a medium containing sodium acetate–acetic acid buffer, pH 3·5 (0·05M) and 3 mg of haemoglobin (horse haemoglobin previously denatured by keeping at pH 1·3 and 37°C for 1 hr). Perchloric acid (0·9 ml, 6·7%) was then added and, after removing precipitated protein by centrifuging, the extinction was measured at 224 nm. An extinction difference of 1 between test and control (homogenate added at end of incubation) was taken as 1 activity unit.

Alkaline proteinase was measured similarly in a medium containing glycine–NaOH buffer, pH 10·0 (0·03M), NaCl (0·075M) and 1 mg of casein (Hammarsten casein, previously dissolved using the minimum amount of NaOH and heated at 100°C for 30 min).

Cathepsins B1 and C and arylamidase were measured using benzoyl-DL-arginine-β-naphthylamide, glycyl-L-phenylalanine-β-naphthylamide and L-leucine-p-nitroanilide, respectively as substrates. Details are given elsewhere [14].

Leucine aminopeptidase was measured in a medium (0·3 ml) containing Tris–HCl, pH 8·2 (0·06M), L-leucine amide (4mM), $MgCl_2$ (5mM) and puromycin (0·1 mM). (Puromycin

was included to inhibit arylamidase, which may also hydrolyse L-leucine amide [15]). Tubes were incubated for 1 hr and 0·3 ml of 10% (w/v) trichloroacetic acid was added; after centrifuging, ammonia was determined by microdiffusion in rotating bottles [16] followed by colorimetric measurement with the indophenol blue reaction. The results (corrected for control tubes) are expressed as μmoles of NH_3 released per hr.

Cathepsin A was assayed using a modification of the method of Iodice *et al.* [17] which measures the release of tyrosine from N-benzyloxycarbonyl – α-L-glutamyl – L-tyrosine. The medium (0·3 ml) contained sodium acetate –acetic acid buffer, pH 5·0 (0·04M) and substrate (0·015M). Tubes were incubated for 3 hr, after which time 0·3 ml of 10% (w/v) trichloroacetic acid was added. The tyrosine present in the supernatant was measured with "Fluram" [18] which reacts with primary amines to form a fluorescent product. ("Fluram" was obtained from Roche Products Ltd., 15, Manchester Square, London, W1M 6AP. An initial gift of this compound from the manufacturers, before it became commercially available, is acknowledged). Samples of the supernatant (0·36 ml) were neutralized with 1·75M NaOH (0·04 ml). The volume was made up to 1·5 ml by addition of 0·2M boric acid–NaOH buffer, pH 9·0, and 0·5 ml of a solution of Fluram in acetone (20 mg/100 ml) was added rapidly, whilst continuously agitating the sample on a Vortex-type mixer. The fluorescence was measured at 475 nm (excitation λ, 390 nm) using an Aminco–Bowman spectrophotofluorimeter. Control incubations without substrate were carried out to correct for the fluorescence due to primary amines present in the homogenate, some of which resulted from endogenous proteolytic activity. To minimize this correction, homogenates were dialysed for 18 hr against water at 4°C before assay. One unit of activity represents 1 nmole hydrolysed per hr.

It has been suggested recently [19] that the low cathepsin A activity reported in some tissues may be due to the fact that basic media were used in the extraction of the enzyme. These authors showed that the activity of cathepsin A in extracts of cerebral grey matter and spleen was much less after the samples were stored at pH 7·5–9·0 than at pH 4·5–7·0. In the present experiment, the activity in muscle homogenates extracted in the usual fashion with 0·04M Tris–HCl, pH 7·6, and dialysed against water, was compared with the activity obtained when 0·01M sodium phosphate, pH 6·5, and 1mM sodium phosphate, pH 6·5 were used as homogenizing medium and dialysis buffer respectively. The activity after alkaline extraction was, in fact, slightly higher, (15%) than after extraction under acid conditions.

Bowen and Davison [19] also reported a thiol-requiring N-benzyloxycarbonyl–α-L-glutamyl– L-tyrosine hydrolase, which could be inhibited by iodoacetate, in extracts of brain and spleen. Addition of iodoacetate (4mM) to the medium in the present experiments had little effect on the release of tyrosine from the substrate, although the endogenous activity of the homogenate was reduced by 20%. Inclusion of EDTA (0·01M) in the assay medium was without effect on cathepsin A activity, thus making it unlikely that metal-containing carboxypeptidases contributed to the measured activity.

Measurement of trypsin-inhibitor activity was based on the method of Reinharz and Roth [20] for assay of serum trypsin inhibitor. Muscle homogenates were centrifuged in a Beckman–Spinco 152 Microfuge for 5 min. Appropriate dilutions of the supernatants were made to give 20–50% inhibition of the hydrolysis of benzoyl-DL-arginine-β-naphthylamide (BANA) by trypsin. The assay medium (0·5 ml) consisted of trypsin (1 μg), BANA (0·2mM), Tris–HCl buffer pH 7·8 (0·05M) and muscle supernatant. Tubes were incubated for 1 hr, after which the β-naphthylamine released was measured as for cathepsin B1. Inhibition was expressed as the difference between the β-naphthylamine released (nmoles/hr) in the presence and absence of muscle supernatant.

The rate of autolysis at pH 3·5 was measured using 0·2 ml of a 1 : 40 muscle homogenate in a medium (total volume 0·3 ml) containing sodium acetate–acetic acid buffer pH 3·5 (0·05M). The release of perchloric acid-soluble products was measured as described for acid proteinase.

All tubes for measurement of enzyme activities were shaken continually at 37°C.

Protein (excluding collagen) in the homogenates was measured [21] after mixing the sample (1 vol) with 9 vol of 0·05N NaOH, allowing it to stand at least 3 hr at room temperature and then centrifuging.

RESULTS

Five series of rats were studied (see Tables). The figures in the tables are means from 6–11 animals. The influence of the implanted tumours upon the body weights of the rats (excluding the weights of the tumours) is shown

Table 1. The effect of Yoshida sarcoma upon the body wt of rats

	Fresh wt (g)					
	Control rats		Tumour-bearing rats		Tumour	Difference*
	Initial	Final	Initial	Final		
Series I–III (20 days)	207·0	271·3	208·2	220·7	74·2	−50·6(±31·7)
Series IV (10 days)	217·4	264·7	215·3	261·0	16·4	− 3·7(±20·5)
Series V (7 days)	191·4	207·8	192·4	211·0	3·0	+ 3·2(±12·5)

*Difference in wt gain (excluding tumour) between control and tumour-bearing rats (±S.D.).

In Series I–III the effect of the tumour upon the wt gain was highly significant ($P < 0·001$).

in Table 1. There was no effect at 7 or 10 days after implantation, but at 20 days the animals with tumours weighed substantially less than the controls. Separate experiments showed that the loss in carcass weight was evident only after 18 days of tumour growth.

During the first 7 days after transplantation, the tumour-bearing animals ate the same amount as the controls (23–25 g/day), but during the next 7 days the average consumption dropped to 6–15% below normal, and from 15–20 days a greater reduction in food intake (20–25%) was observed.

Despite the presence of a tumour of about 25% of the body weight at 20 days, the affected rats were not unduly inactive, although probably less active than the controls. Also, the incapacitation of the left hind limb necessitated extra use of the right hind limb for walking. Thus disuse atrophy of the muscles studied would be extremely unlikely.

Table 2 shows that the weights of the individual muscles were also normal at the earlier time periods, but at 20 days the extensor digitorum longus showed a large weight loss. The soleus, however, remained unchanged. Clark and Goodlad [6] found that the gastrocnemius was affected more than the soleus in rats carrying Walker-256 carcinomas, although the soleus did show some loss of weight As these authors pointed out, the soleus loses less weight than predominantly white muscles in some other conditions of muscle wasting (inadequate food intake, cortisone treatment). After denervation, however, the soleus wastes more rapidly than the extensor digitorum longus [22].

Table 2. The effect of Yoshida sarcoma upon muscle weight and protein in rats

	Extensor digitorum longus			Soleus		
	Control	Mean difference (±S.D.)	P*	Control	Mean difference (±S.D.)	P*
Series I–III (20 days)	97·9	−31·4 (±11·5)	< 0·001	82·8	−0·40(±10·76)	N.S.
	14·63	− 5·5 (± 2·26)	< 0·001	11·41	−0·86(± 2·04)	N.S.
Series IV (10 days)	98·1	− 1·1 (±13·03)	N.S.	83·8	−2·5 (± 3·6)	N.S.
	16·89	+ 0·6 (± 2·30)	N.S.	12·75	+0·51(± 1·62)	N.S.
Series V (7 days)	79·5	− 2·9 (±12·1)	N.S.	71·4	−0·8 (± 6·8)	N.S.
	12·69	− 0·71(± 2·52)	N.S.	10·13	+0·88(± 2·15)	N.S.

*P in this and the following tables refers to the significance of difference between control and tumour-bearing (N.S. = not significant).

In each series, the first line refers to the fresh muscle wt and the second to total muscle non-collagen protein.

The loss of protein in the extensor digitorum longus (Table 2) at 20 days was slightly (7–13%) greater than the fresh weight loss.

The fresh weights of a selected number of organs from several pairs (five) of tumour-bearing and control rats were recorded in Series I. No significant differences were found between the weights of the lungs, brain, heart, or kidneys. The adrenals of tumour-bearing rats have been reported to be enlarged [23]. In the present experiments the adrenals in the host animals were 11% greater than the controls, but not significantly so ($P>0{\cdot}1$). However, the mean liver weight was 34% greater than the control ($P<0{\cdot}001$), and that of the spleen was over 200% greater ($P<0{\cdot}001$).

The specific activities of the enzymes in the muscle homogenates (i.e. activities per unit of muscle protein) are given in Table 3, whilst Table 4 shows the activities per whole muscle. There was no significant change at 7 or 10 days. At 20 days of tumour growth, the specific activities in the extensor digitorum longus of acid proteinase, alkaline proteinase, arylamidase and cathepsin C were increased, whilst that of cathepsin B1 decreased; there were no significant changes in the soleus. Total activities of most of the enzymes in the extensor digitorum longus were decreased. In the soleus there was an increase ($P<0{\cdot}05$) in total leucine aminopeptidase.

DISCUSSION

Increases in specific activity of an enzyme in a wasting condition can evidently result merely from a relative sparing of this enzyme protein. It does not seem likely, therefore, that the increased specific activity of the several enzymes in the extensor digitorum longus muscle in the tumour-bearing animals represents a primary response of the muscle in the presence of the tumour. It is possible, nevertheless, that such increases may, in themselves, cause an increased breakdown of muscle protein, thus accelerating wasting possibly initiated by a

Table 3. The effect of Yoshida sarcoma upon the specific activities of muscle enzymes

	Activity (units/mg muscle protein)					
	Extensor digitorum longus			Soleus		
	Control	Mean difference* (± S.D.)	*P*	Control	Mean difference (± S.D.)	*P*
Series I (20 days)						
Acid proteinase	1·38	+0·54 (±0·41)	< 0·01	1·38	+0·33 (±0·41)	N.S.
Alkaline proteinase	1·46	+0·32 (±0·39)	< 0·05	2·39	+0·11 (±0·42)	N.S.
Cathepsin B1	1·59	−0·44 (±0·47)	< 0·05	2·34	−0·37 (±0·56)	N.S.
Arylamidase	0·250	+0·028(±0·025)	< 0·01	0·392	+0·002(±0·054)	N.S.
Leucine aminopeptidase	0·269	+0·064(±0·113)	N.S.	0·365	+0·135(±0·205)	N.S.
Series II (20 days)						
Cathepsin C	0·122	+0·017(±0·010)	< 0·01	0·184	+0·012(±0·023)	N.S.
Autolysis	0·269	+0·044(±0·099)	N.S.	0·282	+0·091(±0·098)	N.S.
Proteinase inhibitor†	16·0	−2·7 (±7·4)	N.S.	20·3	−4·7 (±6·5)	N.S.
Series III (20 days)						
Cathepsin A	8·67	−1·67 (±2·73)	N.S.	10·63	−0·36 (±2·07)	N.S.
Series IV (10 days)						
Acid proteinase	1·38	+0·05 (±0·15)	N.S.	1·52	−0·07 (±0·29)	N.S.
Alkaline proteinase	1·05	+0·07 (±0·39)	N.S.	1·97	+0·03 (±0·22)	N.S.
Cathepsin B1	1·89	−0·15 (±0·15)	N.S.	2·73	+0·15 (±0·90)	N.S.
Arylamidase	0·201	+0·011(±0·020)	N.S.	0·315	−0·003(±0·052)	N.S.
Series V (7 days)						
Acid proteinase	1·59	+0·05 (±0·22)	N.S.	1·54	−0·02 (±0·32)	N.S.
Alkaline proteinase	1·17	−0·01 (±0·20)	N.S.	2·02	−0·25 (±0·44)	N.S.
Cathepsin B1	2·04	+0·10 (±0·26)	N.S.	2·96	−0·41 (±0·42)	N.S.
Arylamidase	0·225	−0·010(±0·030)	N.S.	0·344	−0·013(±0·060)	N.S.

See text for activity units in each case.
*Increase or decrease in tumour-bearing animals (± S.D.).
†Expressed per mg supernatant protein (see text).

Table 4. The effect of Yoshida sarcoma upon the total activities of muscle enzymes

	Activity (units/muscle)					
	Extensor digitorum longus			Soleus		
	Control	Mean difference (± S.D.)	*P*	Control	Mean difference (± S.D.)	*P*
Series I (20 days)						
Acid proteinase	19·3	− 4·3 (± 3·4)	< 0·01	14·7	+ 2·0 (± 3·3)	N.S.
Alkaline proteinase	19·9	− 4·6 (± 5·0)	< 0·01	25·3	+ 0·1 (± 4·7)	N.S.
Cathepsin B1	25·4	−14·3 (± 6·3)	< 0·001	27·9	− 4·5 (± 9·9)	N.S.
Arylamidase	3·50	− 0·95(± 0·44)	< 0·001	4·34	− 0·23(± 0·88)	N.S.
Leucine aminopeptidase	3·78	− 0·77(± 1·33)	N.S.	3·79	+ 0·88(± 1·13)	< 0·05
Series II (20 days)						
Cathepsin C	1·74	− 0·40(± 0·28)	< 0·01	2·06	− 0·22(± 0·25)	< 0·05
Autolysis	3·55	− 0·75(± 1·05)	N.S.	3·02	+ 0·38(± 1·27)	N.S.
Proteinase inhibitor	71·9	−30·4 (±28·7)	< 0·05	82·3	−24·4 (±32·1)	N.S.
Series III (20 days)						
Cathepsin A	121·6	−60·1 (±46·2)	< 0·001	109·3	− 2·7 (±31·4)	N.S.
Series IV (10 days)						
Acid proteinase	23·2	+ 1·0 (± 3·6)	N.S.	19·3	0 (± 1·9)	N.S.
Alkaline proteinase	17·9	+ 1·1 (± 7·1)	N.S.	25·1	+ 1·2 (± 3·8)	N.S.
Cathepsin B1	30·7	− 1·6 (± 4·3)	N.S.	33·8	+ 3·7 (± 9·9)	N.S.
Arylamidase	3·52	+ 0·08(± 0·40)	N.S.	4·04	+ 0·12(± 0·41)	N.S.
Series V (7 days)						
Acid proteinase	19·7	− 0·80(± 3·2)	N.S.	15·5	+ 1·0 (± 2·4)	N.S.
Alkaline proteinase	14·8	− 1·40(± 3·0)	N.S.	20·3	− 0·6 (± 2·3)	N.S.
Cathepsin B1	25·9	− 0·4 (± 3·3)	N.S.	30·3	− 2·5 (± 3·9)	N.S.
Arylamidase	2·87	− 0·29(± 0·32)	N.S.	3·51	+ 0·18(± 2·09)	N.S.

See Footnotes to Table 3.

decline in protein synthesis. However, more knowledge is required of the factors which control the action of peptide hydrolases in living cells before a definite answer can be given.

An increase in total activity, as seen in the case of leucine aminopeptidase in the soleus, suggests the possibility of a more direct response to the tumour and merits further consideration.

It is of interest that muscle wasting resulting from denervation is associated with marked increase in the total amount of some of these enzymes in the muscle [22]. In particular, cathepsin B1 showed the largest increase in activity after denervation, whereas in the tumour-bearing animals, cathepsin B1 displayed the largest decrease.

It must be emphasized that failure to observe any increase in the total activities of most of these enzymes in the muscle from the tumour-bearing rats does not necessarily exclude the possibility that increased muscle proteolysis results from the presence of the tumour and is a primary cause of the muscle wasting. Such an increase could conceivably result from an effect on the factors (e.g. lysosome stability) which may control the activities of the peptide hydrolases *in vivo*. Such possibilities are under investigation.

It was shown previously [24] that the Yoshida sarcoma gave rise to metastases in the rat, detectable 13 days after implantation into the leg. Since the loss of carcass weight occurred later than this (see above) it is possible that the muscle wasting is related to the dissemination of the tumour.

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REFERENCES

1. A. MAXWELL, Cachexia of malignancy: some possible factors. *New Phycn.* **12,** 452 (1963).
2. C. D. SHERMAN, JR., J. J. MORTON and G. B. MIDER, Potential sources of tumour nitrogen. *Cancer Res.* **10,** 374 (1950).
3. D. J. MILLWARD, Protein turnover in skeletal muscle. I. The measurement of rates of synthesis and catabolism of skeletal muscle protein using [^{14}C] Na_2CO_3 to label protein. *Clin. Sci.* **39,** 577 (1970).
4. 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).
5. E. NORBERG and D. M. GREENBERG, Incorporation of labelled glycine in the proteins of tissues of normal and tumour-bearing mice. *Cancer* **4,** 383 (1951).
6. C. M. CLARK and G. A. J. GOODLAD, Depletion of proteins of phasic and tonic muscles in tumour-bearing rats. *Europ. J. Cancer* **7,** 3 (1971).
7. G. A. J. GOODLAD and C. M. CLARK, Activity of gastrocnemius and soleus polyribosomes in rats bearing the Walker-256 carcinoma. *Europ. J. Cancer* **8,** 647 (1972).
8. G. BENZ and F. E. LEHMANN, Das Verhalten gewebseiniger Proteasen aus normalen und malignen Geweben der Ratte. *Helv. physiol. pharmacol. Acta* **17,** 380 (1959).
9. A. L. BABSON, Catheptic activity in tissues of tumour-bearing rats. *Science* **123,** 1082 (1956).
10. T. SCHERSTEN and K. LUNDHOLM, Lysosomal enzyme activity in muscle tissue from patients with malignant tumour. *Cancer* **30,** 1246 (1972).
11. R. N. FEINSTEIN, Effects of a growth inhibitor and other factors on tissue cathepsins of tumour-bearing rats. *Cancer Res.* **10,** 93 (1950).
12. A. HERZFELD and O. GREENGARD, The differentiated pattern of enzymes in livers of tumour-bearing rats. *Cancer Res.* **32,** 1826 (1972).
13. C. E. BLACKWOOD, G. M. MATEYKO and I. MANDL, Changes in proteolytic enzyme systems of rat tissues in response to heterologous growth of human ovarian tumours. *Cancer Res.* **22,** 993 (1962).
14. A. MONTGOMERY, D. C. PARK and R. J. PENNINGTON, Peptide hydrolases and AMP deaminase in extensor digitorum longus and soleus muscles from genetically dystrophic hamsters. *Comp. Biochem. Physiol.* **49B,** 387 (1974).
15. A. F. BURY and R. J. PENNINGTON, Arylamidases in human muscle. *Biochem. Soc. Trans.* **1,** 1306 (1973).
16. D. SELIGSON and H. SELIGSON, A microdiffusion method for the determination of nitrogen liberated as ammonia. *J. Lab. clin. Med.* **38,** 324 (1951).
17. A. A. IODICE, V. LEONG and I. M. WEINSTOCK, Separation of cathepsins A and D of skeletal muscle. *Arch. Biochem.* **117,** 477 (1966).
18. M. WEIGELE, S. DE BERNADO, J. TENGI and W. LEIMGRUBER, A novel reagent for the fluorimetric assay of primary amines. *J. Amer. chem. Soc.* **94,** 5927 (1972).
19. D. M. BOWEN and A. N. DAVISON, Cathepsin A in human brain and spleen. *Biochem. J.* **131,** 417 (1973).
20. A. REINHARZ and M. ROTH, Studies on the trypsin-like and the trypsin-inhibiting properties of human serum. *Enzym. biol. clin.* **5,** 113 (1965).
21. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
22. M. G. PLUSKAL and R. J. PENNINGTON, Peptide hydrolase activities in denervated muscle. *Biochem. Soc. Trans.* **1,** 1307 (1973).
23. R. W. BEGG, Tumour-host relations. *Advanc. Cancer Res.* **5,** 1 (1958).
24. J. A. DICKSON and H. A. ELLIS, Stimulation of tumour cell dissemination by elevated temperature (42°C) in rats with transplanted Yoshida tumours. *Nature (Lond.)* (In press) (1974).

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Letter to the Editor

Killing of Chinese Hamster Cells *In Vitro* By Heating Under Hypoxic or Aerobic Conditions*

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SEVERAL papers reporting on the heat sensitivity of mammalian cells *in vivo* and *in vitro* have been previously reviewed [1]. In general, the response of mammalian cells to heat contrasts with the response to X-rays. For example, changes in sensitivity during the cell cycle are opposite for heat and X-radiation with S-phase cells being the most radioresistant but the most heat sensitive [2, 3]. Furthermore, in this study, we have found that hypoxia, which makes cells more radioresistant than aerobic cells, does not make them more resistant to heat.

Asynchronous Chinese hamster ovary cells (CHO), (doubling time 12–13 hr), were plated in 14·5 cm^2 glass flasks containing McCoy's 5a medium supplemented with 10% calf and 5% fetal calf serum [2]. The cells were incubated at 37°C in air containing 6% CO_2. Twenty hr after plating, the cells had attained approximately 70% of a monolayer and excess medium was removed, leaving 1 ml which was sufficient to cover the cells.

Hypoxia was induced by placing solid rubber stoppers containing two twenty-gauge needles in the neck of the flasks. The flasks were flushed with water-saturated gas (94% N_2 and 6% CO_2) at a flow rate of 150 cm^3/min. To enhance oxygen effusion from the liquid to the gas phase, the flasks were agitated 100 cycles/min during the 10 min of gassing. A positive N_2 pressure was maintained during removal of the needles. The flasks were maintained at 37°C until heat or radiation treatment.

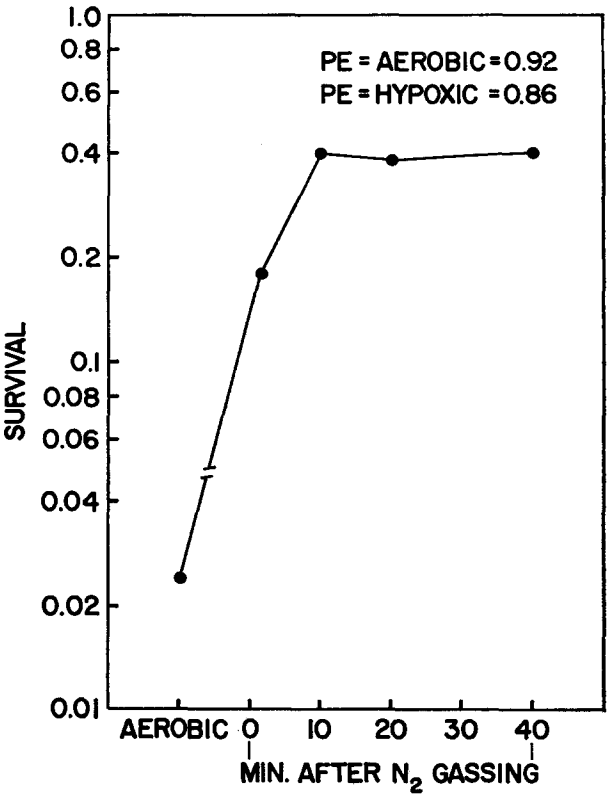


Fig. 1. Log phase CHO cells were given a constant dose of 840 rads under aerobic conditions or at various intervals following 10 min gassing with nitrogen.

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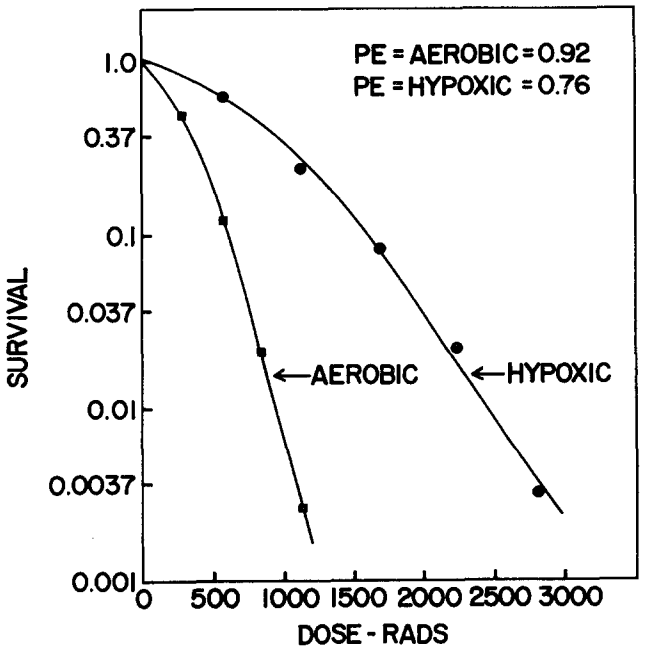


Fig. 2. CHO cells were X-irradiated under aerobic or hypoxic conditions. Hypoxic cells were irradiated 30 min after gassing with nitrogen.

The cells were X-irradiated in their glass flasks at a dose rate of 420 rad/min. The effective energy of the beam was 49 keV. The dose to cells on glass was determined by simultaneously irradiating cells on glass or plastic to known doses for the cells on plastic, and comparing the slopes of their survival curves. Irradiation on glass increased the dose by a factor of 1·4.

Heating was performed by total immersion in a stirred water bath at 45·5°C. The effective heating time at 45·5°C was approximately 0·5 min less than the total immersion time; however, only the total immersion time is reported. We have shown previously [2] that a critical temperature for killing CHO cells does not exist over the temperature range of 43·5 to 46·5°C. This information was obtained from an Arrhenius plot of cell killing versus temperature. A one degree decrease in temperature approximately halved the rate of cell killing. We have also observed pre-heat treatment at 45·5°C eliminates the capacity of cells to accumulate radiation sublethal damage.

Within 2 min after heat or irradiation treatment, 2 ml of medium, at 37°C and saturated with air, was added to the aerobic and hypoxic flasks. Two hours later, the cells were trypsinized, counted and plated in replicate petris. The replicate petris were incubated for 7–10 days at which time the number of colonies containing 50 or more cells was determined and used as an index of survival. Survival was corrected for plating efficiency (PE). Cellular multiplicity was essentially 1 (1–1·09). Each survival point represents the average of 4 replicates.

Figures 1 and 2 show radiobiologic evidence that hypoxia was complete after 10 min of gassing. The oxygen enhancement ratio of 2·5 (Fig. 2) is within the range commonly reported [4]. Heat survival curves determined 30 min after gassing are shown in Fig. 3. Hypoxic cells are at least as sensitive as oxygenated cells, as was previously suggested [5]. The slopes (doses to reduce survival by 37 % on the exponential portion of the survival curves) in Fig. 3 are 1·2 min for hypoxic cells and slightly greater for aerobic cells. Since hypoxic tumour cells, which are relatively radioresistant, may be the cells surviving after radiotherapy [4], the failure of hypoxia to protect cells from heat may be of potential significance in cancer therapy.

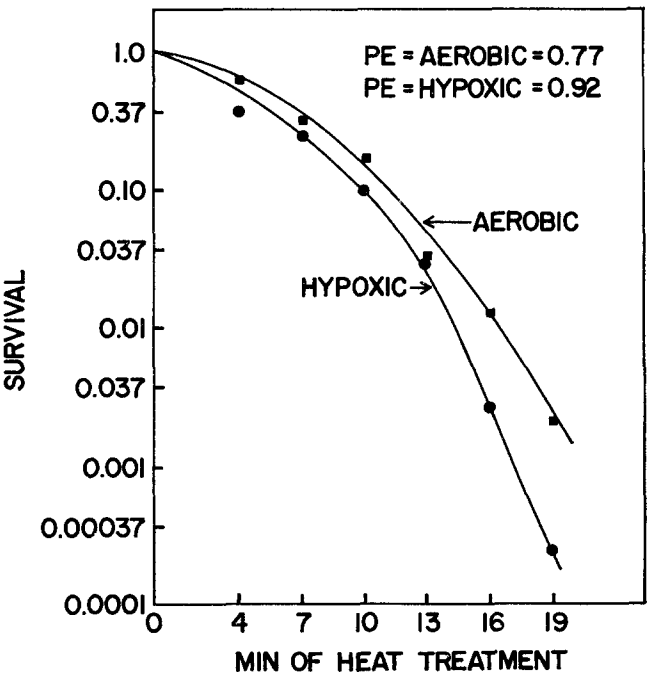


Fig. 3. Glass flasks containing cells were immersed in 45·5°C water for various times as indicated on the abscissa Hypoxia was induced as in Fig. 2.

REFERENCES

1. K. Overgaard and J. Overgaard, Investigations on the possibility of a thermic tumor therapy—I. Shortwave treatment of a transplanted isologous mouse mammary carcinoma. *Europ. J. Cancer* **8,** 65 (1972).

2. A. WESTRA and W. C. DEWEY, Variation in sensitivity during the cell cycle of Chinese hamster cells *in vitro*. *Int. J. Radiat. Biol.* **19,** 467 (1971).
3. R. J. PALZER and C. HEIDELBERGER, Influence of drugs and synchrony on the hyperthermic killing of Hela cells. *Cancer Res.* **33,** 422 (1973).
4. I. F. TANNOCK, Oxygen diffusion and the distribution of cellular radiosensitivity in tumors. *Br. J. Radiol.* **45,** 515 (1972).
5. A. WESTRA, De Invloed Van Straling Op Het Vermogen Tot Proliferatie Van *In vitro* Gekweekte Zoogdiercellen. Ph. D. Thesis, Radiobiological Institute TNO, Rijswijk, The Netherlands, (1971).

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Daunorubicin-DNA: Further Clinical Trials in Acute Non-Lymphoblastic Leukemia

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Abstract—*Treatment with daunorubicin–DNA (DNR–DNA), given as a slow infusion, in combination with small doses of vincristine (VCR) or arabinosine cytoside (ARA-C) has been evaluated in* 19 *patients with acute non-lymphoblastic leukemia. A complete remission was obtained in* 11 *patients* (58%) *with an overall median survival time estimated at* 31 *weeks. The toxicity of DNR–DNA seems to be very low since only one patient (age* 75 *years) died during the induction period. No cardiac toxicity was observed in the four patients who received total doses of DNR–DNA of* 600 *mg/m² or more (up to* 1021 *mg/m²). The main advantage of this therapeutic scheme using DNR–DNA is to induce an excellent remission rate at a low cost of toxicity. It is estimated that the use of lysosomotropic drugs like daunorubicin–DNA is promising and deserves further clinical trials.*

INTRODUCTION

IT WAS shown previously [1] that a complex of daunorubicin (DNR) and DNA is less toxic than free daunorubicin on normal mice and therapeutically more effective on L1210 leukemia inoculated DBA_2 mice. The greater specificity of DNR–DNA is explained by its "lysosomotropism". Unlike free DNR, DNR–DNA does not freely cross the plasma membrane, but is only taken up intracellularly by endocytosis. The complex is then conveyed to the lysosomes where the carrier DNA is digested and DNR released free to diffuse into the cytoplasm and the nucleus. The endocytic properties of many leukemic cells make them very sensitive to this lysosomotropic mode of action while the poor endocytic activity of many normal cells probably explains the lower toxicity of DNR–DNA.

In a series of preliminary clinical trials [2], it was ascertained that DNR–DNA was not affected by a special toxicity and that it was even less toxic than free DNR. Simultaneously, the antileukemic action of DNR–DNA could be demonstrated, even on patients being resistant to free DNR.

This paper gives an outline of the results obtained with DNR–DNA in the treatment of 19 patients suffering from various forms of acute non-lymphoblastic leukemia.

MATERIAL AND METHODS

DNA (calf thymus, type V, or herring sperm, type VII, Sigma, St-Louis) is dissolved at a concentration of 2·34 mg/ml in sterile pyrogen free 0·15 M NaCl. In order to speed the dissolution process, the mixture is heated for 5 min at 120°C and filtered when still warm on a 0·8 μm Millipore filter. The DNA solution is then autoclaved 15 min at 120°C and cooled slowly. In order to avoid pyrogen reactions, it is necessary, according to Lie [3], to autoclave the DNA solution again, 24 hr at most, before preparing and administering the complex.

DNR is dissolved in 0·15 M NaCl at a concentration of 20 mg/ml and added sterilely

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to the DNA solution at a ration of 1 volume of DNR to 100 volumes of DNA. DNR–DNA is administered by an intravenous perfusion at an approximate rate of 100 ml per hr. According to the dose administered, the duration of infusions varies between 3 and 6 hr.

Therapeutic scheme

The protocol used for the induction therapy is given in Table 1. It consists of courses where injections of VCR or ARA-C were followed at 24 hr intervals by injections of DNR–DNA.

Courses were repeated until remission was obtained or resistance became manifest. Depending on the toxicity displayed by VCR the sequential and alternating order of VCR, and ARA-C was not always strictly observed and the number of DNR–DNA perfusions modified with regard to the degree of bone marrow aplasia. Table 1 also indicates the kind of maintenance therapy and the reinduction courses applied to patients in remission.

Patients

Of the 19 patients treated, 16 were new previously untreated cases of acute mon lymphoblastic leukemia. One patient (case 14), suffered a relapse after another type of treatment and two patients suffered from an acute transformation of chronic myeloid leukemia (cases 14 and 15). Details of age, sex and type of leukemia are given in Table 2.

Criteria for remission were those proposed by Hewlett *et al.* [4].

RESULTS

Remission induction

Table 2 also gives the number of treatment courses, total doses of drug and the type of therapeutic response obtained.

In the evaluation of the results, summarized in Tables 3 and 4, all patients were included, even those who underwent an incomplete cycle of therapy. The overall rate of complete remission was 58 % and the best results were obtained in the age group between 15 and 60 years. Complete failure was observed in 3 patients, one of them not having received a complete cycle of therapy.

Total doses of drugs administered varied between 1, 2 and 6 mg/m^2 for VCR, between 100 and 600 mg/m^2 for ARA-C and between 60 and 900 mg/m^2 for DNR–DNA during the induction period.

In one patient (chain 10, Table 2), three of the five courses involved three, and one, four, injections of DRC–DNA because of the apparent resistence of the leukaemic cells. This explains the high total dose of the complex administered during the induction period.

As observed previously [2] DNR–DNA was not affected by a special toxicity. We obtained evidence in favor of a lower toxicity of DNR–DNA. The mucous and gastro-intestinal lesions were rare and never serious and, up to now, no cardiac toxicity was observed in the four patients who received total doses of DNR–DNA of 600 mg/m^2 or more (600, 670, 759 and 1021 mg/m^2), taking into account the doses administered during maintenance therapy in the first four months. On the other hand, when bone marrow aplasia occurred it was easily controlled by current supportive therapy in an open ward and only one patient, the oldest (75 years) died from septicemia during the induction period.

The high risk period of severe therapeutic aplasia can be evaluated by estimating the number and duration of periods during which each patient was in a state of leukopenia and/or thrombopenia with less than 1000 leuko-

Table 1. *Treatment*

	Day 1	Day 2	Day 3	Day 4
Induction				
First course Third course Fifth course ...	VCR 1 mg/m^2 VCR 1 mg/m^2 ←12 hr→	—	DNR–DNA 60 mg/m^2	DNR–DNA 60 mg/m^2
Second course (8–10 days later) Fourth course Sixth course ...	ARA-C 60 mg/m^2 I.v. Injection 1 hr	—	DNR–DNA 60 mg/m^2	DNR–DNA 60 mg/m^2
Maintenance				
at 1, 2, 3, 4, 6 and 9 ... months	VCR 1·5 mg/m^2	—	DNR–DNA 60 mg/m^2	
Weekly	Methyl GAG: 400 mg/m^2			
Daily	6 mercaptopurine: 30–60 mg/m^2			

Table 2. Summary of patients, treatment and evolution

Age groups	No.	Patient Name	Sex	Age (years)	Leukaemia (days)	Duration (days)	Treatment (Induction Period) Courses (number)	Drugs: total doses mg/m² VCR	ARA-C	DRC-ADN	Results after induction	Evolution Duration of C.R. in weeks	Alive (in weeks from start of treatment)	Deceased	Comments
0 to 15	1	DELO	F	3, 5	MBL	23	3	4	200	320	CR	17R	46		No maintenance
	2	MALP	M	3, 5	MBL	27	3	4	200	360	IR			38	Splenomegaly, thrombopenia
	3	THIE	M	4, 5	MBL	39	3	4	200	420	IR		22		Splenomegaly, cutaneous nodules
	4	PLA	M	6, 5	MBL	31	5	6	300	540	CR	4M	11		
15 to 60	5	KRE	F	15	MBL	42	3	4	200	420	CR	45R	59		
	6	BAR	F	21	MBL	31	2	2	200	240	CR	9R		32	
	7	COMB	M	18, 5	MBL	30	3	4	200	456	CR	4		11	Varicella
	8	PIER	M	19	MBL	35	4	2	600	420	IR			26	10% Medullary blasts—Viral hepatitis
	9	ZOL	F	54	PRO	29	2	1, 2	100	180	CR	17R	22		
	10	CHAIN	F	24, 5	UND	49	5	6	400	900	IR			21	32% Medullary blasts
	11	VMEE	M	45, 5	MY-MO	35	4	4	200	600	NR			9	
	12	LEHO	F	45	MY-MO	23	2	2	200	300	CR	17R	26		
	13	SIM	F	46, 5	MONOBL.	35	3	4	200	360	CR	3R		20	Osseous lesions—Cutaneous nodules
	14	ROL	F	19	CML acute	16	2	4	—	300	CR	1		7	Bacterial infection
	15	DUR	F	15	CML acute	3	1	2	—	50	CR	32M	36		
60 and +	16	SAN	F	61	MBL	31	2	2	200	180	NR			26	Septicemia
	17	DOC	F	63	MBL	29	2	1, 2	100	180	CR	17M	22		
	18	THI	M	72	PRO	14	2	1, 2	100	140	IR			16	
	19	DIEP	M	75, 5	PRO	3	1	1	—	60	NR			3	Septicemia

MBL: Myeloblastic
PRO: Promyelocytic
MY-MO: Myelo-Monocytic
UND: Undifferentiated

MONOBL: Monoblastic
CML acute: Acute Transformation of CML

R: Relapsed
M: Maintained

CR: Complete Remilsion
IR: Incomplete Remission
NR: Non Responding

Table 3. *Results*

All leukaemias		Remission rate		
		Complete	Complete + incomplete	No response
Age	Group			
0	15	2/4	4/4	0/4
15	60	8/11	10/11	1/11
60		1/4	2/4	2/4
		11/19 (58%)	16/19 (84%)	3/19 (16%)

cytes/μl and/or less than 20,000 platelets/μl. Six of the 19 patients were never below 1000 leukocytes during the induction period. Ten patients experienced one leucopenic period ranging from 3 to 17 days with a mean length of 7·5 days; 2 patients had two leucopenic periods of 1 and 4 days respectively, and one patient had 3 leucopenic periods of approximately 4 days each. Two patients were in such a profound state of thrombopenia at the start of treatment that their period of high risk cannot be evaluated. Five other patients never went lower than 20,000 platelets/μl and 11 patients each had one thrombopenic period varying between 1 and 19 days with a mean length of 7·5 days. One patient experienced two thrombopenic periods of 3 and 14 days length.

Long term results

From the 11 patients in complete remission, two died in complete remission from viral or bacterial infection, while 6 of the 9 remaining relapsed after various periods (Table 2).

The actuarial survival curve for all the patients, given in Fig. 1, shows a median survival time of 31 weeks. In a preceding series of 19 patients where induction therapy was performed by our group according to Crowther *et al.* [5] using ARA-C and DNR, we observed a complete remission rate of 43% and a median survival of 9 weeks. The difference between the two survival curves lies essentially in the early mortality rate while the results at 50 weeks are very comparable.

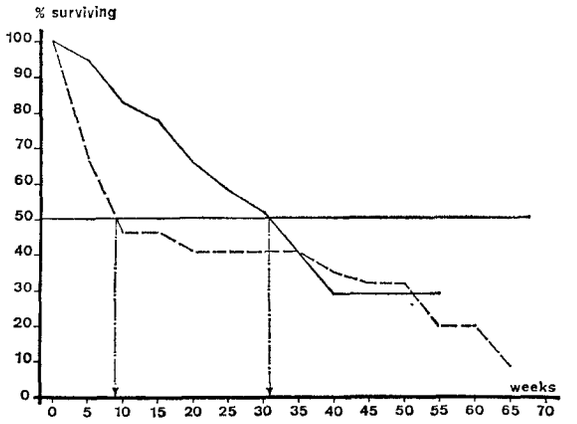


Fig. 1. *Actuarial survival curves of non lymphoblastic leukemia patients treated in our department.* ———: *19 patients treated with DNR-DNA.* – – – –: *19 patients treated with DNR and ARA-C according to Crowther* et al. [5].

DISCUSSION

The principal aim of our clinical trial was to learn more about the handling of DNR–DNA, to study its toxicity and to evaluate its therapeutic efficiency on the basis of the rate of remissions obtained. Therefore, we decided not to perform a randomized trial with DNR and DNR–DNA because of the high heterogeneity of the acute non lymphoblastic leukemias and their relative rarity which would have made a long series of patients necessary and a long time to complete it. Moreover, in order to give the greatest chance to the patients and in view of the bad prognosis of non lymphoblastic leukemia we thought it more ethical to give the drug in combination with VCR and or ARA-C. These drugs were given, at doses which, when given alone, would not have a major effect on the leukemic process. VCR and ARA-C were given 24 hr before the DNR–DNA perfusion in the hopes of obtaining a synchronization of the leukemic cell populations.

The rate of 58% complete remission is among the best obtained in medicine today and this is even more striking when one

Table 4. *Complete remissions according to age and type of leukemia*

Age	Group	Myelobl. + Promyeloc.	Myelo and/or Monocytic	Acute transformation	Undifferentiated
0	15	2/4			
15	60	4/5	2/3	2/2	0/1
60		1/4			
		7/13	2/3	2/2	0/1

considers the great dispersion of the patients' ages. This result was obtained at a low cost of toxicity. Only one patient (75 years old) died during the induction period and this compares very favorably with the rate of mortality of $\pm 30\%$ observed with the best therapeutic regimens known to give about 50% complete remissions.

Although, it is difficult to draw definite conclusions from our small series, with regard to the response rate of different leukemia types, it should be emphasized that acute transformation of chronic myeloid leukemia seems to be very sensitive to DNR–DNA. The same sensitivity was observed with adriamycin–DNA in two other patients not reported here. We recommend therefore, that only half doses be used in this type of leukemia and that the degree of aplasia be carefully determined before pursuing the treatment.

Serious mucous or gastro-intestinal complications were not observed, marrow aplasia, when it occurred, was easily controlled in an open ward.

The duration of the induction period was approximately 30 days and the high risk period of profound aplasia (< 1000 leukocytes and $< 20{,}000$ platelets/μl) limited to ± 5 days. To date, no cardiac toxicity has been observed in any of our patients. This involves both the acute toxicity during the induction period and the chronic cardiac toxicity due to the cumulative effect of repeated doses, although four patients received more than 600 mg/m^2 in less than four months, which is the critical dose considered to be fraught with a high risk [6, 7].

In our personal experience with a series of patients treated with free DNR, we observed a cardiac toxicity in 8 patients who received more than 600 mg/m^2 DNR (range 617–1103; mean value 775 mg) adminstered in a longer period, in 12 months. It should be noted that lesser cardiac toxicity of DRC–DNA vs free DRC was otherwise recently demonstrated by an experimentation on isolated perfused heart preparation [8].

The medium survival time of 31 weeks is much longer than that of 9 weeks which we observed in one of our previous series of patients; it is in the range of the survival times obtained by other authors in the treatment of non lymphoblastic leukemia. The main advantage of our protocol using DNR–DNA is to induce remissions of non lymphoblastic leukemia at a very good rate with a very low cost of toxicity. However the maintenance of the remission obtained still remains a problem. We think that by adjusting the sequence of DNR–DNA administration during the induction period (5 days consecutive administrations every 15–20 days instead of 2 weekly perfusions) and by increasing the reinduction dosage it will be possible not only to improve the rate of complete remission but also to increase substantially the survival time.

For a better understanding of the action of the drug, we have finally to consider that in the use of the DRC–DNA complex, the viscosity of the preparation imposes slow infusion as compared with the common rapid injection of free DRC. Although randomization between free and complexed drug has not as yet been performed on humans, the slow infusion of the complex does not explain, in our opinion, a difference in action. First, this randomization was indeed done in animals [1], and lesser toxicity with higher therapeutic activity was demonstrated for the complex. Secondly, recent pharmacological studies [9] concerning the plasma levels of the free and the complexed drug, infused at the same rate in humans, clearly demonstrates that the complex has a much longer half-life and more sustained plasma levels. In this sense the DNA moiety, and not the duration of infusion, modifies the pharmacology of the drug.

Certainly many problems remain to be resolved, namely the pharmacology of DNR–DNA and its optimal dosage and rhythm of administration. In our opinion, the use of lysosomotropic drugs like DNR–DNA seems very promising and justifies further clinical trials as well as further fundamental and experimental work.

REFERENCES

1. A. Trouet, D. Deprez-De Campeneere and C. de Duve, Chemotherapy through lysosomes with a DNA–daunorubicin complex. *Nature (New Biol.)* **239,** 110 (1972).
2. G. Sokal, A. Trouet, J. L. Michaux and G. Cornu, DNA–daunorubicin complex: preliminary trials in human leukaemia. *Europ. J. Cancer* **9,** 391 (1973).
3. S. Lie, (Personal communication).

4. J. S. Hewlett, J. D. Battle, R. C. Bishop, W. M. Fowler, S. O. Schwartz, P. S. Hagen, J. Louis, The midwest co-operative chemotherapy study group. Phase II study of A 8103 (NSC 25.154) in acute leukemia in adults. *Cancer Chemother. Rep.* **42,** 25 (1964).
5. D. Crowther, C. J. T. Bateman, C. P. Vartan, J. M. A. Whitehouse, J. S. Nalpas, G. Hamilton Fairley and R. Scott, Combination chemotherapy using L-asparaginase, daunorubicin, and cytosine arabinoside in adults with acute myelogenous leukaemia. *Brit. med. J.* **4,** 513 (1970).
6. B. Jones, J. F. Holland, A. R. Morrison, S. L. Lee, L. F. Sinks, J. Cuttner, A. Rausen, F. Kung, H. J. Pluss, F. I. Haurani, R. B. Patterson, J. Blom, E. O. Burgert, Jr., J. H. Moon, L. Chevalier, A. Sawitsky, M. Albala, R. J. Forcier, G. Falkson and O. Glidewell, Daunorubicin (NSC 82151) in the treatment of advanced childhood lymphoblastic leukemia. *Cancer Res.* **31,** 84 (1971).
7. J. Bernard, C. Jacquillat and M. Weil, Treatment of the acute leukemias. *Seminars in Hematology*, **9,** 181 (1972).
8. A. Langslet, I. Øye and S. Lie, Decreased cardiac toxicity of Adriamycine and Daunorubicine when bound to DNA. *Biochem. Pharmacol.* (In press).
9. A. Trouet and R. Hulhoven, (Personal communication).

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Eradication of Tumour Cells by Successive Injections of Allogeneic Immune and Hyperimmune Peritoneal Cells in a Murine Lymphoma System*

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Abstract—*Allogeneic C57BL immune and hyperimmune (vs SL2) peritoneal cells are used for eradication of DBA/2 derived SL2 lymphoma cells injected into the peritoneal cavity of DBA/2 mice.*

SL2 bearing DBA/2 mice are treated with 3, 5, or 8 successive i.p. injections of 2×10^6 allogeneic C57BL immune or hyperimmune peritoneal macrophages (contaminated with lymphocytes). 2×10^7 SL2 cells could be eradicated successfully with five injections of immune or three injections of hyperimmune C57BL peritoneal cells. This is a ten-fold improvement compared to the result obtained with single injections of immune or hyperimmune peritoneal cells.

The number of injections with immune or hyperimmune cells, time intervals between the injections and the total length of the period of the therapy was important for the result of the therapy.

INTRODUCTION

ALLOGENEIC immune and hyperimmune peritoneal exudate cells, comprising about equal numbers of macrophages and lymphocytes, are very effective tools in immunotherapy of ascitic lymphoma cells injected into the syngeneic host [1–3]. With one injection of 2×10^6 immune or hyperimmune peritoneal macrophages, contaminated with an approximately equal number of lymphocytes, 2×10^6 SL2 cells can be eradicated.

In this paper, experiments are described in which SL2 bearing DBA/2 mice are treated with successive i.p. injections of allogeneic immune or hyperimmune peritoneal cells. With this technique, even 2×10^7 SL2 cells can be eradicated. The importance of this result is clear if this number of cells is compared to the maximum number of cells that can be obtained from a transplantation mouse which is 5×10^8 SL2 cells. Furthermore, it has always been stressed that with immunotherapy, only a very small number of tumour cells, no more than 10^3–10^5 cells, can be eradicated [4–6].

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MATERIAL AND METHODS

Mice

Pure bred DBA/2, C57BL and CBA mice, 6–10 weeks old were used. DBA/2 mice were used as: (a) a source of DBA/2 derived SL2 lymphoma cells; (b) SL2 bearing mice to be cured; and (c) as a source of DBA/2 liver cells. C57BL mice were used as a source of allogeneic non-immune, immune or hyperimmune peritoneal, spleen and lymphnode cells. CBA mice

were used as a source of CBA derived TLC5 lymphoma cells.

Lymphomas

The DBA/2 derived lymphoma SL2, and the CBA derived lymphoma TLC5 were maintained by weekly i.p. passage.

Both lymphomas were gifts from Dr. P. Alexander.

Immunization

C57BL mice were immunized by one or four i.p. injections of 10^7 SL2, 10^7 TLC5 or 10^7–10^8 DBA/2 liver cells.

Immunization schedules

(a) C57BL mice were immunized by one i.p. injection of 10^7 SL2, 10^7 TLC5 or 10^7–10^8 DBA/2 liver cells. Peritoneal cells were harvested 10 days later. This is the optimal time for collection of allogeneic immune peritoneal cells [1, 2]. (b) C57BL mice were hyperimmunized by four injections at days 0, 9, 18 and 27. The peritoneal cells were collected 5 days after the last immunization. (This is the optimal time for collection of allogeneic hyperimmune peritoneal cells [3]).

Collection of peritoneal cells

Peritoneal cells (non-immune, immune or hyperimmune) were collected by washing the peritoneal cavity with 5 ml Fischer's medium. Cells were centrifuged and resuspended in Fischer's medium [7].

Cells used for therapy

(a) 2×10^6 immune or hyperimmune (vs. SL2) allogeneic C57BL peritoneal macrophages/ml. The macrophages were contaminated with an approximately equal number of lymphocytes [1, 2].

Cells used as controls

(a) 2×10^6 immune or hyperimmune (vs TLC5) allogeneic C57BL peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes [1, 2]. (b) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL peritoneal lymphocytes/ml. These lymphocytes were freed of macrophages by twice exposing the exudate to glass for 30 min at 37°C. Lymphocytes were contaminated with less than 7% macrophages as counted in a haemocytometer. (c) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL peritoneal macrophages/ml. These macrophages were freed of lymphocytes by injecting 0·3 ml anti lymphocyte serum (ALS)† i.p. into immune or hyperimmune C57BL mice 45 min before collection of the peritoneal cells. Macrophages were contaminated with less than 8% lymphocytes as counted in a haemocytometer. (d) 2×10^6 non-immune (normal) C57BL allogeneic peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes. (e) 2×10^6 non-immune allogeneic C57BL peritoneal macrophages/ml, freed of lymphocytes (see also c). Macrophages were contaminated with less than 7% lymphocytes as counted in a haemocytometer. (f) 2×10^6 non-immune allogeneic C57BL peritoneal lymphocytes/ml, freed of macrophages (see also b). Lymphocytes were contaminated with less than 5% macrophages. (g) 2×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL lymph node cells/ml. Lymphoid cells were obtained from the lymph-nodi mandibulares. Lymph nodes were cut in Fischer's medium. (h) 2×10^6 non-immune allogeneic C57BL lymph node cells/ml (see also g). (i) 4×10^6 immune or hyperimmune (vs SL2) allogeneic C57BL lymphoid cells of the spleen/ml. Spleen cells were obtained by rinsing out the spleen by injecting 5 ml of Fischer's medium. (j) 4×10^6 non-immune allogeneic C57BL lymphoid cells of the spleen/ml. (k) 2×10^6 immune or hyperimmune (vs DBA/2 liver) C57BL peritoneal macrophages/ml. Macrophages were contaminated with lymphocytes.

RESULTS

Therapy with immune peritoneal cells

The results of the first series of experiments are summarized in Table 1. It was shown that treatment of SL2 bearing mice with 5 i.p. injections of immune C57BL peritoneal cells was most effective in eradication of tumour cells. Two out of five mice survived a dose of as much as 2×10^7 SL2 cells i.p. for more than 35 days. Treatment of SL2 bearing mice with 8 injections of allogeneic immune peritoneal cells was less successful than with 3 or 5 injections.

Therapy with hyperimmune peritoneal cells

The effect of treatment of SL2 bearing mice with 3 or 5 i.p. injections of 2×10^6 C57BL hyperimmune (vs SL2) peritoneal macrophages (contaminated with lymphocytes) is shown in Table 2. Two out of five DBA/2 mice treated with 3 i.p. injections of hyperimmune cells

†ALS: ALS was a horse-anti mouse serum. It was a gift from Radiobiological Institute, TNO, Rijswijk, The Netherlands.

Table 1. *Effect of successive* injections with* 10 *days immune (vs SL2) allogeneic C57BL peritoneal cells into SL2 bearing DBA/2 mice*

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment					
No. of SL2 cells	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7	10^8
Treatment with						
3 i.p. injections of immune peritoneal cells†	5/5 (5 > 150)	4/5 (59,3 > 150)	3/5 (3 > 150)	4/5 (4 > 150)	0/5 (10·2 ± 0·2)	0/5 (8·6 ± 1·4)
3 i.p. injections of Fischer's medium only	0/5 (19·0 ± 0·0)	0/5 (18·0 ± 0·0)	0/5 (16·0 ± 0·0)	0/5 (15·0 ± 0·0)	0/5 (9·0 ± 0·0)	0/5 (8·0 ± 1·0)
5 i.p. injections of immune peritoneal cells†	4/5 (35·3 > 150)	3/5 (39, 59, 1 > 150)	2/5 (2 > 150)	3/5 (37, 40, 1 > 150)	2/5 (2 > 150)	0/5 (9·0 ± 0·0)
5 i.p. injections of Fischer's medium only	0/5 (13·0 ± 0·0)	0/5 (10·5 ± 0·5)	0/5 (10·0 ± 0·0)	0/5 (10·0 ± 0·0)	0/5 (9·0 ± 0·0)	0/5 (8·0 ± 0·0)
8 i.p. injections of immune peritoneal cells†	2/5 (35, 51)	1/5 (72)	0/5 (23·6 ± 4·5)	2/5 (38, 1 > 150)	0/5 (14·2 ± 1·2)	Not done
8 i.p. injections of Fischer's medium only	0/5 (14·6 ± 0·8)	0/5 (13·6 ± 0·5)	0/5 (13·6 ± 0·8)	0/5 (11·0 ± 0·0)	0/5 (10·0 ± 0·0)	Not done

*The first injection was given 2 hr after challenge, the following injections were given with time intervals of 24 hr.
†2×10^6 allogeneic immune peritoneal C57BL macrophages contaminated with an approximately equal number of lymphocytes; the immune cells were collected 10 days after immunization.

Table 2. *Effect of successive* i.p. injections with hyperimmune (vs SL2) allogeneic C57BL peritoneal cells into SL2 bearing DBA/2 mice*

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment			
No. of SL2 cells	2×10^5	2×10^6	2×10^7	10^8
Treatment with				
3 i.p. injections of hyperimmune peritoneal cells†	4/5 (38, 44, 2 > 150)	3/5 (42,2 > 150)	2/5 (35,1 > 150)	0/5 (10·4 ± 1·0)
3 i.p. injections of Fischer's medium only	0/5 (11·5 ± 0·5)	0/5 (11·5 ± 0·5)	0/5 (10·0 ± 1·0)	0/5 (10·5 ± 0·5)
5 i.p. injections of hyperimmune peritoneal cells†	4/5 (60,3 > 150)	3/5 (38,2 > 150)	0/5 (12·8 ± 1·6)	Not done
5 i.p. injections of Fischer's medium only	0/5 (14·0 ± 0·0)	0/5 (11·5 ± 0·5)	0/5 (8·5 ± 0·5)	Not done

*The first injection was given 2 hr after challenge, the following injections were given with time-intervals of 24 hr.
†2×10^6 allogeneic peritoneal macrophages from C57BL mice immunized at day 0, 9, 18, 27; the cells were collected 5 days after the last immunization, macrophages were contaminated with an approximately equal number of lymphocytes.

survived a dose of 2×10^7 SL2 cells for more than 35 days. When treated with 5 i.p. injections with hyperimmune peritoneal cells, only a dose of 2×10^6 SL2 cells could be eradicated: three out of five mice survived more than 35 days after challenge.

Therapy with 3 *successive injections with immune or hyperimmune peritoneal cells within* 24 *hr after challenge*

SL2 bearing DBA/2 mice were treated with 3 successive i.p. injections of 2×10^6 allogeneic C57BL immune or hyperimmune peritoneal macrophages (contaminated with lymphocytes) 2, 13, and 24 hr after challenge. The number of survivors at 35 days after challenge decreases considerably (Table 3).

When DBA/2 mice (not injected with SL2 cells) were treated with 3 i.p. injections of 2×10^6 immune C57BL peritoneal macrophages (contaminated with lymphocytes) within 24 hr, 1 out of 5 mice died. However, this was not caused by the allogeneic cells, but was due to proliferating SL2 cells present among the 10 days immune cells [1, 2],

Three i.p. injections hyperimmune peritoneal cells within 24hr caused no harm to the recipients.

Comparison of the 3 *foregoing series of experiments*

Comparison of the 3 foregoing series of experiments (Table 4) shows that treatment with 5 i.p. injections of allogeneic *immune*

*Table 3. Effect of 3 successive i.p. injections of allogeneic immune or hyperimmune (vs SL2) C57BL peritoneal cells into SL2 bearing DBA/2 mice within 24 hr**

	Number of survivors at 35 days (real survival time in days in parentheses) after treatment				
No. of SL2 cells	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7
Treatment with					
3 i.p. injections of immune peritoneal cells†	1/5 (1 > 150)	0/5 (21·6 ± 0·8)	0/5 (22·2 ± 5·2)	0/5 (21·2 ± 2·9)	0/5 (10·6 ± 1·0)
3 i.p. injections of hyperimmune peritoneal cells†	Not done	Not done	4/5 (49, 3 > 150)	0/5 (24·4 ± 3·2)	0/5 (15·4 ± 2·7)
3 i.p. injections of Fischer's medium only	0/5 (15·0 ± 0·0)	0/5 (14·0 ± 1·0)	0/5 (12·5 ± 0·5)	0/5 (11·8 ± 0·4)	0/5 (9·8 ± 0·4)

*The injections were given 2, 12, and 24 hr after challenge with SL2 cells.

†2×10^6 immune or hyperimmune peritoneal C57BL macrophages contaminated with lymphocytes.

Table 4. Comparison of the results of the three foregoing series of experiments as summarized in Tables 1, 2 and 3

	Number of survivors at 35 days after challenge		
No. of SL2 cells	2×10^5	2×10^6	2×10^7
Treatment with			
3 i.p. injections* of immune peritoneal cells†	3/5	4/5	0/5
5 i.p. injections* of immune peritoneal cells†	2/5	3/5	2/5
8 i.p. injections* of immune peritoneal cells†	0/5	2/5	0/5
3 i.p. injections* of hyperimmune peritoneal cells†	4/5	3/5	2/5
5 i.p. injections of hyperimmune peritoneal cells†	4/5	3/5	0/5
3 i.p. injections‡ of immune peritoneal cells† within 24 hr	0/5	0/5	0/5
3 i.p. injections‡ of hyperimmune peritoneal cells† within 24 hr.	4/5	0/5	0/5

*The first injection was given 2 hr after challenge with SL2 cells, the rest of the injection was given with time intervals of 24 hr.

†2×10^6 immune or hyperimmune C57BL peritoneal macrophages contaminated with lymphocytes.

‡The injections were given 2, 13 and 24 hr after challenge.

peritoneal cells, or with 3 i.p. injections of allogeneic *hyperimmune* peritoneal cells gives the best results in eradication of tumour cells. Treatment of SL2 bearing DBA/2 mice with 3 i.p. injections with immune or hyperimmune peritoneal cells within 24 hr after challenge was less successful.

Control cell suspensions

Experiments in which the control suspensions were tested showed that only immune or hyperimmune peritoneal lymphocytes were able to eradiacate 2×10^5 SL2 cells. When DBA/2 mice were challenged with 2×10^5 SL2 cells and treated with 3 successive i.p. injections with 2×10^6 C57BL immune or hyperimmune peritoneal lymphocytes 1 out of 5, and 3 out of 5 mice respectively survive for more than 35 days (Table 5). However, when DBA/2 mice were injected i.p. with 2×10^6 SL2 cells and treated with 3 i.p. injections of immune or hyperimmune peritoneal lymphocytes, none of the mice survived for more than 35 days. Further, only treatment with 3 successive i.p. injections of 4×10^6 C57BL hyperimmune lymphoid cells of the spleen, and 2×10^6 C57BL hyperimmune peritoneal macrophages caused a prolongation of life time of the SL2 bearing DBA/2 mice. With the rest of the control suspensions, only a very slight prolongation of lifetime or no prolongation at all was obtained.

DISCUSSION

It is well established that resistance to allograft murine tumours can be transferred by immune or hyperimmune peritoneal cell suspensions [2, 3, 8–11]. Transfer of immunity by immune [12–14] or hyperimmune lymphocytes [3] and hyperimmune spleen cells [3, 4] has been reported. It has been shown also that passive transfer of allogeneic immuno-competent lymphoid cells under certain conditions may hold in check experimental tumours temporarily and sometimes even destroy the tumour completely [15–18].

The experiments described in this paper show that treatment of SL2 bearing DBA/2 mice with successive i.p. injections of allogeneic immune or hyperimmune peritoneal cells is very successful (Tables 1, 2).

Table 5. *Effect of* 3 *successive* i.p. injections of C57BL control cell suspensions into DBA/2 mice injected i.p. with* 2×10^5 *SL* 2† *cells*

Cell suspension	Number of survivors at 35 days (real survival time in parentheses) after treatment		
	Non-immune	Immune	Hyperimmune
2×10^6 peritoneal lymphocytes	0/5 (12·0 ± 1·6)	1/5 (42)	3/5 (36, 37, 1 > 150)
2×10^6 peritoneal macrophages	0/5 (13·2 ± 0·4)	0/5 (15·0 ± 0·6)	0/5 (17·6 ± 2·6)
2×10^6 lymph node cells	0/5 (12·6 ± 0·5)	0/5 (12·8 ± 0·8)	0/5 (13·0 ± 0·0
4×10^6 spleen cells	0/5 (12·8 ± 0·4)	0/5 (15·4 ± 1·1)	0/5 (27·0 ± 3·3)
2×10^6 peritoneal macrophages contaminated with lymphocytes	—	0/5‡(12·2 ± 0·7)	0/5‡(15·4 ± 2·2)
2×10^6 peritoneal macrophages contaminated with lymphocytes	—	0/5§(12·2 ± 0·7)	0/5§(11·2 ± 0·4)
2×10^6 peritoneal macrophages contaminated with lymphocytes	0/5 (12·2 ± 0·5)	—	—

*injection were given 2, 24 and 48 hr after injection SL2 cells.

†controls, injected with 2×10^5 SL2 cells only died after 12·0 ± 0·0 days.

‡immune, or hyperimmune vs CBA derived TLC5 lymphoma.

§immune, or hyperimmune vs DBA/2 liver cells.

The number of SL2 cells that could be eradicated, however, did not correlate simply with the number of injections with allogeneic immune or hyperimmune peritoneal cells. Treatment of SL2 bearing DBA/2 mice with 8 i.p. injections of allogeneic immune peritoneal cells reduced significantly the number of survivors at 35 days after challenge.

This reduction possibly might be due to a sensitization reaction of the DBA/2 host caused by the successive administration of allogeneic cells [12] resulting in rejection of the immune cells. Rejection of these allogeneic immune cells may lead to a "free" proliferation of SL2 lymphoma cells which might still be present in the immune peritoneal cell suspension obtained from mice immunized 10 days earlier [1, 2], and which might finally kill the mouse. Den Otter *et al.* [19] were able to show that only 1 SL2 cell i.p. is sufficient to kill a DBA/2 mouse within 26 days.

Development of a severe Graft vs Host reaction eventually resulting in early death of the test animals, as described by Woodruff *et al.* [17, 18] was not noticed as all mice which died within 35 days after challenge had a fully developed tumour.

A significant reduction of the number of survivors at 35 days after challenge was also shown when SL2 bearing DBA/2 mice were treated with 3 i.p. injections of immune or hyperimmune peritoneal cells within 24 hr after challenge. However, treatment of DBA/2 mice (not injected with SL2 cells) with 3 i.p. injections of immune or hyperimmune peritoneal cells within 24 hr caused no harm to the recipients. Further investigation on this subject will be necessary.

In control experiments, it was shown that only treatment of DBA/2 mice, injected i.p. with 2×10^5 SL2 cells by 3 successive i.p. injections of 2×10^6 *immune* or *hyperimmune* C57BL peritoneal lymphocytes (contaminated with 7 % macrophages) resulted in survival of 1 out of 5, and 3 out of 5 mice respectively, for more than 35 days after challenge (Table 5). However, when DBA/2 mice were challenged with 2×10^6 SL2 cells, there were no survivors at 35 days. Further, only treatment with hyperimmune spleen cells resulted in a significant prolongation of life-time, but there were also no survivors at 35 days.

These results approve earlier findings [1–3]: (a) optimal therapeutic effects in this allogeneic system are obtained when a suspension of peritoneal macrophages contaminated with lymphocytes is used, and (b) the reaction described in this paper may be regarded as immunologically specific as treatment with peritoneal cells from C57BL mice immunized or hyperimmunized against CBA derived TLC5 lymphoma, or against DBA/2 liver cells did not result in significant prolongation of life.

REFERENCES

1. H. F. J. Dullens, and W. Den Otter, Eradication of lymphoma cells with allogeneic immune peritoneal cells. *Experientia* (*Basel*) **29,** 479 (1973).

2. H. F. J. Dullens and W. Den Otter, Therapy with allogeneic immune peritoneal cells. *Cancer Res.* **34,** 1726 (1974).
3. H. F. J. Dullens, F. J. Kingma and W. Den Otter, Immunotherapy with allogeneic hyperimmune peritoneal cells in a murine lymphoma system. *Europ. J. Cancer* **10,** 41 (1974).
4. P. Alexander, Prospects for immunotherapy of cancer: experience in experimental systems. *Brit. med. J.* **4,** 484 (1970).
5. P. Alexander, D. 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).
6. G. Mathé, Immunological treatment of leukemias. *Brit. med. J.* **4,** 487 (1970).
7. G. A. Fischer, Studies of the culture of leukemia *in vitro*. *Ann. N.Y. Acad. Sci.* **76,** 673 (1958).
8. D. B. Amos, In *Mechanism of Cell and Tissue Damage Production by Immune Reactions. II. International Symposium on Immunopathology.* (Edited by P. Grabar and P. Miescher), Schwabe, Basel (1962).
9. P. Baker, R. S. Weiser, J. Jutila, C. E. Evans and R. J. Blandau, Mechanisms of tumor homograft rejection: the behavior of sarcoma I ascites tumor in the A/Jax and C57BL/6K mouse. *Ann. N.Y. Acad. Sci.* **101,** 46 (1962).
10. B. Bennett, Specific suppression of tumour growth by isolated peritoneal macrophages from immunized mice. *J. Immunol.* **95,** 656 (1965).
11. W. E. Hoy and D. S. Nelson, Studies on Cytophylic antibodies. V. Alloantibodies cytophylic for mouse macrophages. *Aust. J. exp. Biol. med. Sci.* **47,** 525 (1969).
12. H. Borberg, H. F. Oettgen, K. Choudry and E. J. Beattie, Jr., Inhibition of established transplants of chemically induced sarcomas in syngeneic mice by lymphocytes from immunized donors. *Int. J. Cancer* **10,** 539 (1972).
13. E. J. Delorme and P. Alexander, Treatment of primary fibrosarcoma in the rat with immune lymphocytes. *Lancet* **18,** 117 (1964).
14. B. Klein, H. O. Sjögren, E. Klein and K. E. Hellström, Demonstration of resistance against methylcholantrene induced sarcomas in the primary autochtonous host. *Cancer Res.* **20,** 1561 (1960).
15. L. Ellman, D. H. Katz, I. Green, W. E. Paul and B. Benacerraf, Mechanisms involved in the antileukemic effect of immunocompetent allogeneic lymphoid cell transfer. *Cancer Res.* **32,** 141 (1972).
16. D. H. Katz, L. Ellman, W. E. Paul. I. Green and B. Benacerraf, Reistance of guinea pigs to leukemia following transfer of immunocompetent allogeneic lymphoid cells. *Cancer Res.* **32,** 133 (1972).
17. M. F. A. Woodruff and M. O. Symes, The use of immunologically competent cells in the treatment of cancer. Experiments with a transplantable mouse tumour. *Brit. J. Cancer* **16,** 707 (1962).
18. M. F. A. Woodruff and J. L. Boak, Inhibitory effect of pre-immunized CBA spleen cells on transplants of L-strain mouse mammary carcinoma in (CBA × A) F_1 hybrid recipients. *Brit. J. Cancer* **19,** 411 (1965).
19. W. Den Otter, E. A. Runhaar, C. A. Ruitenbeek and H. F. J. Dullens, Site-dependent differences in rejection of tumour cells with and without preimmunization. *Europ. J. Immunol.* **4,** 444 (1974).

Europ. J. Cancer Vol. 10 pp. 707–712. Pergamon Press 1974. Printed in Great Britain.

Uptake of Progesterone-^{14}C and Estradiol-^{3}H by Isolated Normal Mammary Parenchymal and Cancer Cells of the Rat*

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Abstract—*By utilizing the enzyme collagenase, it was possible to obtain intact normal mammary parenchymal and mammary cancer cells. The isolated cells were incubated for 2 hr with either 0·2 μCi(1·04 μg)progesterone-4-^{14}C or 0·2 μCi(1·4 ng) 17β-estradiol-6,7-^{3}H. The results demonstrated that both progesterone and estradiol are preferentially incorporated into hormone responsive cancer cells to a greater extent than that of normal mammary parenchymal cells.*

INTRODUCTION

IT IS WELL established that ovarian hormones will greatly affect the induction and growth of chemically induced mammary cancers. Jull [1], as well as Huggins *et al.*, [2], demonstrated that ovariectomized animals fed polycyclic hydrocarbons exhibited a decreased incidence and an increased time of appearance of the first palpable mammary cancer when compared to intact animals receiving such carcinogens. The administration of progesterone has been shown by Jabara and Harcourt [3] to induce mammary neoplasms in 25 % of ovariectomized rats receiving 9,10-dimethyl-1,2-benzanthracene(DMBA). Furthermore, progestational states (pregnancy and lactation) have similarly been shown to increase the induction and growth of many DMBA-induced mammary cancers [4, 5].

The binding of estradiol to specific nuclear and cytoplasmic proteins in normal and neoplastic mammary tissue of both the human and the rat is well established. However, due to the lipid solubility of steroids, a quantitative comparison of the selective uptake and binding of estradiol by these tissues has not been demonstrated. Furthermore, although progesterone has been shown to influence the growth of mammary tissue and some chemically induced mammary tumors, very few studies have been conducted relative to the selective uptake and binding of this steroid [6, 7]. Hence, it was of interest to determine if the DMBA-induced mammary cancer cells selectively incorporate these steroids and, if so, whether binding is as great or less than that of normal mammary parenchymal cells.

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MATERIAL AND METHODS

The purity of DMBA, 17β-estradiol-6,7-^{3}H (40 Ci/mmole), and progesterone-4-^{14}C (61 mCi/mmole) was established by thin layer chromatography. Sprague–Dawley female rats received a single oral feeding of 20 mg DMBA dissolved in 1 ml of sesame oil at 50 days of age. Beginning four weeks after DMBA feeding, the animals were palpated and the detectable tumors measured three times each week to establish rapidly growing neoplasms. When the tumor, regardless of its anatomical position, reached a size approximating 16 × 22 mm, the ovaries of the rat were removed. If the tumor regressed within the following 4–7 days to approximately 25 % of its size prior to ovariectomy, it was classified as a hormone responsive tumor and was utilized in these studies.

The animals bearing hormone responsive tumors were lightly anesthetized with ether and sacrificed by decapitation. The abdominal–inguinal mammary glands on one side of the animal and the tumor were rapidly excised and placed in sterile petri dishes. A portion of the tumor was placed in neutral formalin and later processed for histological classification. The mammary gland was separated into a parenchymal cell fraction and a fat cell fraction by the procedure of Moon *et al.*, [8]. Only a slight modification of the method for obtaining intact mammary parenchymal cells was required to isolate cancer cells. Tumor tissue was cut from areas which were grossly free of any necrotic tissue. The quantity of collagenase (Worthington Biochemical Corp., Code: CLS, Freehold, New Jersey) was increased to 70 mg per 1 g tissue because of the slightly greater quantity of connective tissue in the adenocarcinoma as compared to mammary tissue. In addition, the time of incubation was increased to 2 hr. Since these cells have been grown in tissue culture, it is apparent that the collagenase treatment did not result in cellular damage. The washed cells were placed in 50 ml Erlenmeyer flasks containing 1 ml medium 199, 0·8 ml 2·8 % sodium bicarbonate, 0·2 ml penicillin–streptomycin mixture, 8 ml sterile water and either 0·2 μCi (1·04 μg) progesterone-4-^{14}C or 0·2 μCi (1·4 ng) 17β-estradiol-6,7-^{3}H. Incubation was performed for 2 hr at 37° with slow agitation. The cells were washed 3 times with saline and then placed in 10 ml of 2 :1 chloroform–methanol for 24 hr to extract the intracellular lipid. This extraction procedure separated the cells into an intracellular lipid fraction and a dry, fat-free tissue (DFFT) fraction which were analyzed for either radioactivity, total lipid, or DNA content.

The dissolution and counting of tissues were performed by the method of Janss and Moon [9]. Actual counts for all samples were at least 5 times above background. The intracellular lipid of the isolated mammary parenchymal and cancer cells were extracted by the method of Folch *et al.*, [10]. DNA was extracted from the dry, fat-free tissue residues according to Schneider [11], while DNA content was determined by the colorimetric procedure of Webb and Levy [12]. The Student's *t*-test was employed for determining the difference between means and any differences were considered to be statistically significant if the level of confidence was 95 % or greater.

RESULTS

The specific activity of the intracellular lipid (Fig. 1) extracted from mammary tumor cells (110×10^2 dis/min mg^{-1} lipid) exposed to estradiol-6,7-^{3}H greatly exceeded that of normal mammary parenchymal cells (42×10^2 dis/min mg^{-1} lipid). The radioactive content of the tumor cell was also found to be statistically greater than that of the mammary parenchymal cell DFFT. The specific activity of the intracellular lipid fraction of tumor cells incubated with progesterone-4-^{14}C did not differ statistically from that obtained from normal mammary paren-

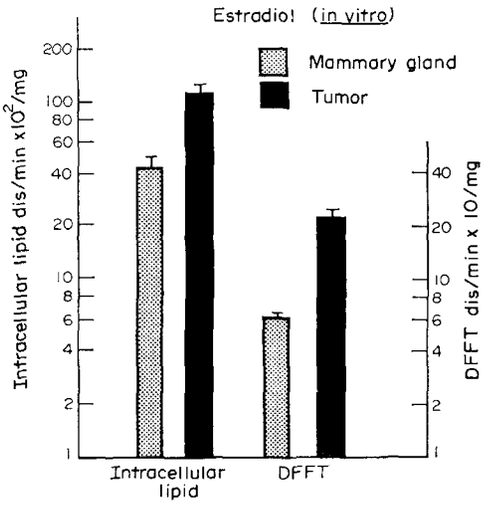


Fig. 1. Comparison of the uptake of estradiol-6,7-^{3}H by isolated mammary parenchymal and DMBA-induced mammary tumor cell fractions obtained from ovariectomized rats. The intact cells were incubated for 2 hr at 37° with 0·2 μCi (1·4 ng) estradiol-6,7-^{3}H. Each bar represents the mean specific activity ± SE (dpm/mg of either intracellular lipid or dry, fat-free tissue) of cell fractions obtained from at least 5 different animals.

chymal cells (Fig. 2). However, the specific activity of the DFFT from mammary cancer cells was more than twice that found in the parenchymal cell DFFT.

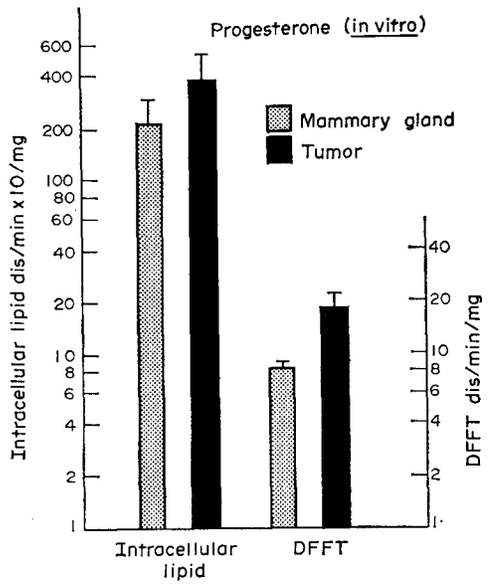


Fig. 2. Comparison of the uptake of progesterone-4-^{14}C by isolated mammary parenchymal and DMBA-induced mammary tumor cell fractions obtained from ovariectomized rats. Experimental protocol was the same as that described in the legend of Fig. 1, except that cells were incubated with 0·2 μCi (1·04 μg) progesterone-4-^{14}C.

Table 1 compares the total lipid and DNA content of mammary parenchymal and regressing tumor cells from rats which were ovariectomized 4 to 7 days prior to isolation of the cells. Similar quantities of isolated cells were obtained when 1·5 g of mammary gland and 0·6 g of tumor tissue were treated with collagenase. The intracellular lipid content of the mammary parenchymal cells was approximately 1·1 % of the cellular wet weight and did not differ statistically from the lipid content of the tumor cells. Furthermore, DNA analysis of the isolated cells revealed that the DNA content of normal parenchymal cells was similar to that of cells obtained from DMBA-induced cancers that regress after ovariectomy.

DISCUSSION

Both normal and neoplastic human breast tissue were reported by Deshpande *et al.*, [6] to accumulate and retain estradiol-6,7-^{3}H for up to five hours following an i.v. injection of the hormone. The concentration of estradiol in the neoplastic tissue was three times greater than that observed in normal breast tissue. However, other studies [13, 14] have shown that a selective uptake of estradiol does not occur in all human mammary cancers. Korenman and Dukes [15] found that only 5 of 15 patients having either infiltrating ductal carcinomas or adenocarcinomas exhibited a higher estrogen binding capacity than that of the uninvolved mammary gland tissue. A similar uptake of estradiol by hormone dependent and independent mammary tumors induced by DMBA has been demonstrated in the rat [7, 16–18].

Hilf *et al.*, [19] have determined that infiltrating ductal carcinomas of the human mammary gland contain one-third the triglyceride level of normal breast tissue. Since steroids are lipid soluble, a quantitative comparison of the uptake and binding of ovarian steroids by normal and neoplastic mammary tissues has not been previously undertaken. However, the method of Moon *et al.*, [8] makes such a study feasible since it

Table 1. Total lipid and DNA content of isolated mammary parenchymal and DMBA-induced mammary cancer cells

Tissue fraction	Wet weight (mg)	Total lipid (mg)	Per cent total lipid*	DNA (μg) Isolated cells Wet weight (mg)
Mammary gland				
Collagenase-treated	1486·6 ± 86·6			
Isolated cells	48·1 ± 8·7	0·52 ± 0·09	1·17 ± 0·10	1·044 ± 0·241
Mammary cancer				
Collagenase-treated	621·6 ± 28·6			
Isolated cells	47·2 ± 6·3	0·46 ± 0·11	1·12 ± 0·04	1·179 ± 0·294

*Milligrams of total lipid per milligram of isolated cells times 100.
All values are means ± SE of at least five different animals whose mean body weight was 278·6 ± 5·4 g.

effectively separates the interfering fat cells from the parenchymal cells.

The concentration of estradiol-6,7-^{3}H in the intracellular lipid fraction of cancer cells was significantly greater than that within the same fraction of normal mammary parenchymal cells. Since a difference in intracellular lipid content could account for the higher specific activity in the neoplastic cells, the percentage of intracellular lipid of the isolated cells was also determined. However, the lipid content of both the intact normal and neoplastic mammary parenchymal cells was approximately 1·1% of their cellular wet weight. Thus, the greater concentration of estradiol in the intracellular lipid of cancer cells apparently resulted from either an increased transport of this steroid through the cell membrane and/or a greater solubility of estradiol in this fraction of tumor cells.

Numerous investigators [14, 20–24] have reported the binding of estradiol to nucleic acids and cellular proteins in normal and malignant mammary tissue. In the present study, the binding of estradiol by DNA, RNA and/or cellular proteins, as indicated by the specific activity of the dry, fat-free residue, was much greater in neoplastic cells than in normal mammary parenchymal cells.

Mobbs [7] failed to find a difference in the uptake of subcutaneously injected progesterone by hormone responsive DMBA-induced mammary tumors and indicated that binding of this steroid does not occur in these tumors. However, binding sites or receptors for progesterone have been identified in the uterus [25], chick oviduct [26] and the prostate [27]. Following the identification of progesterone receptors in the rat and rabbit uterus, McGuire and DeDella [25] suggested that tissues which respond to steroid hormones must contain receptors which are stereospecific for these hormones. Since the mammary gland and many DMBA-induced mammary cancers do respond to progesterone, it follows that these tissues probably also contain progesterone receptors and should show a selective binding of the hormone. Indeed, a recent study by Terenius [28] has suggested that progesterone receptors do exist in the human and DMBA-induced mammary cancer.

Our data revealed that the level of progesterone-4-^{14}C uptake by the intracellular lipid of tumor cells did not differ statistically from that found in normal mammary parenchymal cells. This is in contrast to the greater uptake of estradiol-6,7-^{3}H by the intracellular lipid of tumor cells. However, the binding of progesterone-4-^{14}C to the dry, fat-free residue of neoplastic cells was more than twice that occurring in the normal mammary parenchymal cells. The greater binding of progesterone-4-^{14}C and estradiol-6,7-^{3}H to isolated mammary cancer cells agrees well with that seen in *in vivo* studies [29].

The use of hormonal therapy has been used widely since Beatson [30] reported the beneficial effect of ovariectomy in a woman suffering from breast cancer. Paradoxically, the administration of exogenous estrogen and/or progesterone will cause the regression of some mammary cancers in the human [31–33]. The observations that receptors for estradiol have been identified in human breast cancers and the importance of the receptor in predicting hormone dependency of such tumors is currently of great interest. Maass *et al.*, [34] Leung *et al.*, [35] and Engelsman *et al.*, [36] found that patients who were positive for estradiol receptor sites exhibited an objective remission to various endocrine therapy, while patients lacking these receptors did not respond to endocrine treatment. It is obvious from this that knowledge of specific steroid binding can be useful in prescribing further therapy for breast cancer patients. Although progesterone receptors have not been isolated in either the rat or human mammary cancer, the present data and that of Terenius [28] strongly suggest their existence. Hopefully, the procedure described can be used clinically to quantitatively compare the uptake of estradiol, progesterone and other steroids by normal and neoplastic cells of breast cancer patients.

SUMMARY

A quantitative comparison of estradiol and progesterone uptake by isolated normal and neoplastic mammary cells has not been possible previously due to the rapid uptake of these steroids by the fat cells of the normal mammary gland. However, by utilizing the enzyme collagenase, it was possible to obtain intact mammary parenchymal and cancer cells. The isolated cells were incubated with either progesterone-4-^{14}C or estradiol-6,7-^{3}H for 2 hr at 37°C. The specific activity of the intracellular lipid of normal and malignant cells did not differ following incubation of the cells with progesterone-4-^{14}C. In contrast, the concentration of estradiol-6,7-^{3}H in the intracellular lipid of tumor cells was significantly greater than that of normal mammary parenchymal cells. Binding of both estradiol-6,7-^{3}H and

progesterone-4-^{14}C to the lipid-extracted residue of mammary cancer cells was greater than that of normal parenchymal cells. Hopefully, the procedure described can be used clinically to quantitatively compare steroid uptake by normal and neoplastic cells of breast cancer patients.

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REFERENCES

1. J. W. Jull, The effect of oestrogens and progesterone on the chemical induction of mammary cancer in mice of the IF strain. *J. Path. Bact.* **68,** 547 (1954).
2. C. Huggins, G. Briziarelli and H. Sutton, Rapid induction of mammary carcinoma in the rat and the influence of hormones on the tumors, *J. exp. Med.* **109,** 25 (1959).
3. A. G. Jabara and A. G. Harcourt, The effect of progesterone and ovariectomy on mammary tumors induced by 7,12-dimethylbenzanthracene in Sprague–Dawley rats. *Pathology* **2,** 115 (1970).
4. C. Huggins, R. C. Moon and S. Morii, Extinction of experimental mammary cancer. I. Estradiol-17β and progesterone. *Proc. natl. Acad. Sci.* **48,** 379 (1962).
5. G. M. McCormick and R. C. Moon, Effect of pregnancy and lactation on growth of mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA). *Brit. J. Cancer* **19,** 160 (1965).
6. N. Deshpande, R. D. Bulbrook and F. O. Belzar, An apparent selective accumulation of progesterone by the human breast. *Proc. Second Int. Cong. Hormonal Steroids,* 750 (1966).
7. B. G. Mobbs, The uptake of simultaneously administered (^{3}H) oestradiol and (^{14}C) progesterone by dimethylbenzanthracene-induced rat mammary tumors. *J. Endocr.* **41,** 339 (1968).
8. R. C. Moon, D. H. Janss and S. Young, Preparation of fat cell-"free" rat mammary gland. *J. Histochem. Cytochem.* **17,** 182 (1969).
9. D. H. Janss and R. C. Moon, Uptake and clearance of 9,10-dimethyl-1,2-benzanthracene-9-^{14}C by mammary parenchymal cells of the rat. *Cancer Res.* **30,** 473 (1970).
10. J. Folch, M. Lees and G. H. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues. *J. biol. Chem.* **226,** 497 (1957).
11. W. C. Schneider, Phosphorus compounds in animal tissues I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161,** 293 (1945).
12. J. M. Webb and H. B. Levy, A sensitive method for the determination of deoxyribonucleic acid in tissues and microorganisms. *J. biol. Chem.* **213,** 107 (1955).
13. P. Feherty, G. Farrer-Brown and A. E. Kellie, Oestradiol receptors in carcinoma and benign disease of the breast: An *in vitro* assay. *Brit. J. Cancer,* **25,** 697 (1971).
14. H. Johansson, L. Terenius and L. Thoren, The binding of estradiol-17β to human breast cancer and other tissues *in vitro*. *Cancer Res.* **30,** 692 (1970).
15. S. G. Korenman and B. A. Dukes, Specific estrogen binding by the cytoplasm of human breast carcinoma. *J. clin. Endocr.* **30,** 639 (1970).
16. S. Sander and A. Attramadal, The *in vivo* uptake of oestradiol-17β by hormone responsive and unresponsive breast tumors of the rat. *Acta path. microbiol. Scand.* **74,** 169 (1968).
17. L. Terenius, Selective retention of estrogen isomers in estrogen-dependent breast tumors of rats demonstrated by *in vitro* methods. *Cancer Res.* **28,** 328 (1968).
18. 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. Wood, Jr.) p. 15. The University of Chicago Press, Chicago and London (1967).
19. R. Hilf, H. Goldenberg, I. Michel, R. A. Orlando and F. L. Archer, Enzymes, nucleic acids and lipids in human breast cancer and normal breast tissue. *Cancer Res.* **30,** 1874 (1970).

20. R. Hahnel, E. Twaddle and A. B. Vivian, Estrogen receptors in human breast cancer. II. *In vitro* binding of estradiol by benign and malignant tumors. *Steroids* **18,** 681 (1971).
21. R. J. B. King, J. Gordon, D. M. Cowan and D. R. Inman, The intranuclear localization of (6,7-^{3}H)-oestradiol-17 β in dimethylbenzanthracene-induced rat mammary adenocarcinoma and other tissues. *J. Endocr.* **36,** 139 (1966)
22. J. L. Wittliff, R. Hilf, W. F. Brooks, Jr., E. D. Savlov, T. C. Hall and R. A. Orlando, Specific estrogen-binding capacity of the cytoplasmic receptor in normal and neoplastic breast tissues of humans. *Cancer Res.* **32,** 1983 (1971).
23. W. L. McGuire, Estrogen receptors in human breast cancer. *J. clin. Invest.* **52,** 73 (1973).
24. B. S. Leung, L. C. Manaugh and D. C. Wood, Estradiol receptors in benign and malignant disease of the breast. *Clin. chim. Acta* **46,** 69 (1973).
25. J. L. McGuire and C. DeDella, *In vitro* evidence for a progestogen receptor in the rat and rabbit uterus. *Endocrinology* **88,** 1099 (1971).
26. B. W. O'Malley, M. R. Sherman and D. O. Toft, Progesterone "receptors" in the cytoplasm and nucleus of chick oviduct target tissue. *Proc. natl. Acad. Sci.* **67,** 501 (1970).
27. R. Karsznia, R. H. Wyss, W. L. Heinrichs and W. L. Herrman, Binding of pregnenolone and progesterone by prostatic "receptor" proteins. *Endocrinology* **84,** 1238 (1969).
28. L. Terenius, Estrogen and progesterone binders in human and rat carcinoma. *Europ. J. Cancer* **9,** 291 (1973).
29. C. J. Grubbs, Reproductive hormones and 9,10-dimethyl-1,2-benzanthracene-induced mammary carcinogenesis in the rat. Ph.D. Dissertation, University of Tennessee Medical Units, 1973.
30. G. T. Beatson, On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet* **2,** 104 (1896).
31. D. Gordon, B. N. Horwitt, A. Segaloff, P. J. Murison and J. V. Schlosser, Hormonal therapy in cancer of the breast. III. Effect of progesterone on clinical course and hormone excretion. *Cancer* **5,** 275 (1952).
32. B. J. Kennedy, Effect of massive doses of estradiol undecylate in advanced breast cancer. *Cancer Chemother. Rep.* **51,** 491 (1967).
33. F. M. Muggia, P. A. Cassileth, M. Ochoa, F. A. Flatow, A. Gellhorn and G. A. Hyman, Treatment of breast cancer with medroxyprogesterone acetate. *Ann. intern. Med.* **68,** 328 (1968).
34. H. Maass, B. Engel, H. Hohmeister, F. Lehmann and G. Trams, Estrogen receptors in human breast cancer tissue. *Amer. J. Obstet. Gynec.* **113,** 377 (1972).
35. B. S. Leung, W. S. Fletcher, T. D. Lindell, D. C. Wood, and W. W. Krippaehne, Predictability of response to endocrine ablation in advanced breast carcinoma. *Arch. Surg.* **106,** 515 (1973).
36. E. Engelsman, J. P. Persijn, C. B. Korsten, F. J. Clefton, Oestrogen receptor in human breast cancer tissue and response to endocrine therapy. *Brit. med. J.* **2,** 750 (1973).

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A Kinetic Analysis of Death and Survival in HeLa Cells Following Exposure to Methylazoxymethanol Acetate*

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Abstract—*The technique of time lapse microcinematography has been used to follow the fate of the progeny of HeLa cells exposed to methylazoxymethanol (MAM acetate) at dose levels which allow some degree of survival. The effects of the drug are minimal during the treatment cycle, but are pronounced during the subsequent cycle. An extended interphase occurs and most cell death is related to the mitosis following this interphase. Chromosome analysis, performed at the time of this mitosis, has shown the presence of aberrations of varying severity. The relationship between these lesions and cell death is discussed.*

INTRODUCTION

THE CARCINOGENIC activity in mammals of methylazoxymethanol and its more stable acetate (MAM acetate) is well established [1–3]; these agents are also mutagenic in bacteria [4] and drosophila [5]. Matsumoto and Higa [6] have shown that MAM acetate is able to methylate nucleic acids at the N7 position of guanine.

The effects of this DNA methylating agent upon the progress of cells through the cell cycle is complex. The studies of Van den Berg and Ball [7], in our laboratories, showed that the extent of the disturbance of HeLa cell kinetics depended on the phase of the cell cycle in which the cells were exposed to MAM acetate. Using doses that allowed only 5% survival (as measured by colony formation), they observed that DNA synthesis was maximally inhibited in the 2nd S phase after treatment, that is, when cells treated in G1 or S had divided once and cells treated in G2 had divided twice. N-methyl-N-nitrosourea (MNU), another DNA methylating agent, has a similarly delayed action on DNA synthesis in HeLa S_3 cells [8].

Both groups of workers have used biochemical methods to investigate the consequences of the damage to the DNA; this has given valuable information about the cell population as a whole but is unable to indicate the behaviour of individual cells. We have examined the fate of individual HeLa S_3 cells and their progeny, after treatment with MAM acetate, using continuous microcinematography. This work was undertaken for two reasons; firstly to examine the action of MAM acetate with particular reference to the recovery and death of cells, and secondly, to use it as a model system to devise methods of examining the effects of drugs that may have lethal effects that are not apparent for a few generations after exposure to the agent.

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MATERIAL AND METHODS

Cells

The HeLa S_3 cell line, a line identical to that used by Van den Berg and Ball [7] and obtained originally from the Chester Beatty Research Institute, was maintained in exponential phase in Eagle's Minimal Essential Medium, either in monolayer or as suspension cultures. The Minimal Essential Medium (MEM) was supplemented with foetal calf serum (10 or 7%), non-essential amino acids and glutamine, all obtained from either Flow or Biocult Labs. The MEM was buffered with bicarbonate except for filming purposes, when this was substituted with HEPES buffer (20 mM) [9].

Synchronization and treatment with MAM acetate

Synchronous cultures were prepared by the mitotic selection technique of Robbins and Marcus [10], as described previously [7]. Cells were exposed to MAM acetate (20 $\mu g\text{-}cm^{-3}$ final concentration) for 1 hr immediately prior to mitotic harvest; a treatment in G2 or to MAM acetate (100 $\mu g cm^{-3}$) 3 hr after shake off—a mid-G1 treatment. These doses were chosen to compare and contrast the effects of a high and low dose, as it had previously been demonstrated that they allowed survivals of 70% and 5% respectively, as measured by the ability of cells to form colonies [7].

Following exposure, the drug was removed by centrifugation (800 rev/min for 3 min). The cell pellet was washed twice with fresh medium and resuspended in MEM (HEPES buffered).

Time lapse cinematography

Modified Rose chambers were inoculated with cells at $0{\cdot}5\text{–}1{\cdot}0 \times 10^5$ cells cm^{-3}. The chambers were incubated at 37°C using a heated water jacket and viewed with a Zeiss inverted microscope, fitted with a long focal length phase contrast optics (objective ×10, eye piece ×4). Time lapse cinematographic films were made with an exposure of 1 sec at 1 min-intervals using a Vinten intervalometer and mark III camera. The cell cycle during which all filming commenced is referred to as the 1st cycle, and subsequent cycles are classified accordingly. This terminology is shown in Fig. 1. The films were analyzed frame by frame, either using a Steenbeck editing table or a Specto Mark III analysing projector.

Chromosome analysis

Duplicate synchronous cultures were set up at the same time as those filmed by time lapse cinematography. Four ounce medical flats were inoculated with MEM containing cells at $0{\cdot}5 \times 10^5$ cells cm^{-3}. Cells were allowed to settle, become attached and were incubated at 37°C. At various times after treatment, monolayers were harvested with a "rubber policeman", treated hypotonically with 0·8% sodium citrate and fixed in methanol/acetic acid (3 : 1). After removing the fixative and gently resuspending the cells in a few drops of 70% acetic acid, the cells were spread onto breath-moistened slides and stained with proprionic orcein.

Cytological examination of intact mitotic figures

Drops of about 0·2 cm^3 of both the treated and control synchronous cell suspensions ($0{\cdot}5 \times 10^5$ cells cm^{-3}) were carefully placed onto microscope slides in plastic Petri dishes. Following attachment, the microscope slides were immersed in MEM (HEPES buffered) and incubated at 37°C.

The slides were removed from the Petri dishes at various time intervals, rinsed in Earle's balanced salt solution and fixed for 10 min in methanol/acetic acid (3 : 1). The slides were subsequently dried and stained with azure A.

Scanning electron microscopy

Two cubic centimeters of both the treated and control synchronous cell suspensions, ($0{\cdot}5 \times 10^5$ cells cm^{-3}) were added to 4 cm Petri dishes containing coverslips. The Petri dishes were subsequently incubated at 37°C.

At various time intervals between 50–100 hr after treatment, the coverslips were processed for scanning electron microscopy. Following a brief rinse in warm Earle's balanced salt solution, the coverslips were fixed by immersion in 2·5% glutaraldehyde in sodium cacodylate buffer (pH 7·4, osmolarity 360 mosmoles kg^{-1}) for 60 min.

Following fixation, the samples were dehydrated in a series of graded acetone solutions and critical point dried from liquid carbon dioxide. The samples were fixed to aluminium stubs with electrically conductive adhesive, coated with gold palladium to a thickness of about 50 nm and examined with a Stereoscan 600 scanning electron microscope (Cambridge Scientific Instruments Ltd.).

RESULTS

The HeLa cells used had a main line of hypodiploid cells and a secondary line of hypotetraploid cells (< 15%). The control cultures also contained a few multinucleate cells. It is probable that the formation of the cells of higher ploidy was a secondary event arising from instability in the stem line. The low frequency of the higher ploidy cells suggests they gained no biological advantage over the stem line.

The kinetics of untreated cells

The average cycle time for untreated cells used in this study was 1100–1400 min. The mean durations of interphase (taken as the time between successive periods of rounding up for mitosis) and the mean durations of mitosis, for 39 first generation cells and their progeny, monitored on three films for 4 generations, are shown in Table 1.

Table 1. *Cell cycle times for synchronous control HeLa cells*

Cycle	No. cells*	Interphase time† (min)	Mitotic time† (min)
1	39	1192 ± 45	47 ± 4
2	78	1130 ± 29	50 ± 3
3	100	1128 ± 33	60 ± 3
4	113	1485 ± 80	69 ± 6

*Cells were selected as being suitable for analysis on the film—not all cells present remained within the frame throughout the period of observation.
†Mean ± S.E.M.

In the control films, recording a total of 330 cells, cell death was less than 1% and no multipolar divisions were seen, although they could be found in larger samples studied cytologically. The increase of both interphase and mitotic times in the 3rd and 4th cycles may be associated with confluence and subsequent depletion of the medium. The initially high synchronization of 1st generation cells (> 90% at shake off) was subject to a decay with time, although partial synchrony was present in the 4th cycle.

The kinetics of cells exposed in G1 to MAM acetate (100 $\mu g\ cm^{-3}$)

The mean interphase and mitotic times for 53 cells and their surviving progeny, following exposure to MAM acetate in the G1 phase, were analysed from films made continuously for six days. The results are the aggregates of 3 films and are shown in Table 2. The values obtained relate to survivors in each cycle, although their progeny did not necessarily survive subsequent cycles.

Table 2. *Cell cycle times for synchronous HeLa cells exposed to* 100 μgcm^{-3} *MAM acetate during the first G1 period following synchronization by mitotic selection**

Cycle	Number of cells observed	Interphase time† (min)	Mitotic time† (min)
1	53	1105 ± 50	54 ± 7
2	54	2816 ± 157	180 ± 36
3	24	2000 ± 175	79 ± 11
4	22	1375 ± 57	79 ± 20

*A cell is included if it completes the cycle. Cells dying during interphase or mitosis are excluded.
†Mean ± S.E.M.

By comparing these results with the corresponding values for untreated cells, it can be seen that there was a marginal prolongation of interphase in the 1st cycle, but the 1st mitosis was normal in duration. The extension of the 2nd interphase was pronounced and the 2nd mitosis was also prolonged. This alteration of kinetics was less evident in the 3rd cycle and was no longer exhibited by survivors during the 4th cycle.

An increase in the incidence of tripolar divisions was observed but this was restricted to the 2nd mitosis. The majority of these divisions were followed by cell fusions and inevitably by the death of the fused cells. For analytical purposes, such tripolar divisions, followed by fusions were regarded as divisions giving rise ultimately to two new cells.

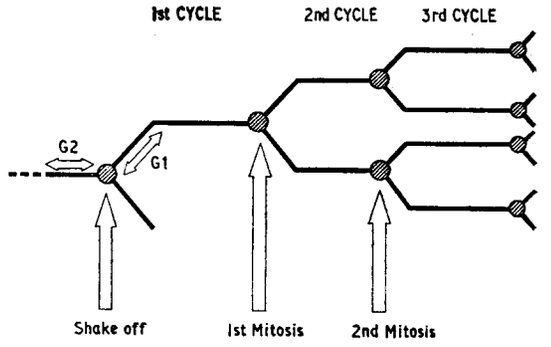


Fig. 1. *Classification of cell cycles following initial treatment with MAM acetate. Filming commenced during the 1st cycle.*

Cell death

The final stages of cell death in all the films examined was characteristic; this being a period of vigorous expulsion and withdrawal of large protrusions of the cytoplasm. Price [11] has used the term zeiosis to describe such activity and Bessis [12] has emphasized its relationship with impending cell death. Cells exhibiting this surface activity were also identified in samples examined by scanning electron microscopy (Fig. 2–3). For this analysis, the time of cell death was taken to have occurred at the commencement of this phenomenon, as it had a very distinct appearance and was constant in duration.

There was no cell death in the first cycle and all the cells monitored passed into the 2nd cycle. Eight per cent of the cells entering the 2nd cycle neither divided nor died during the six days of observation. They were monitored on average, for 7958 ± 524 min and were considered in this analysis as "non-cycling" cells. Similarly, the fate of 20 % of the cells in the 3rd cycle could not be ascertained, as they had neither divided nor died by the 6th day when filming ceased. They remained in this "non-cycling" state for an average of 5108 ± 495 min.

The majority of cell death occurred in the 2nd and 3rd cycles, and the distribution of survival, death and "non-cycling" cells in each cycle, is shown in Fig. 4.

The cinematographic analysis showed that the events prior to cell death were of two patterns. Firstly, there were those cells that rounded up, became refractile and appeared similar to cells in mitosis, but remained in this rounded, refractile state for many hours and then died. The time at which these cells died was, in the 2nd cycle, 4108 ± 257 min from the beginning of the cycle. The majority of cell death in the 2nd cycle was of this type. The timing of the commencement of this pattern of death, i.e. the initial rounding up, was also associated with the time when survivors of that cycle were seen to enter mitosis and successfully divide.

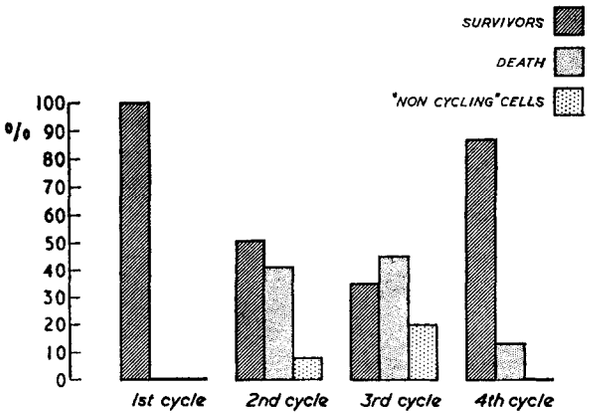


Fig. 4. Distribution of death and survival, in each cycle following exposure to MAM acetate (100 μgcm^{-3} *G1 treatment).*

The other pattern of cell death was seen predominately in the 3rd cycle. At 773 ± 42 min after mitosis, cells which had flattened out and were indistinguishable from other cells in interphase, exhibited the terminal sequence of cell disruption without any prior period of rounding up. This pattern was also seen in the 2nd cycle (753 ± 116 min from mitosis) but accounted for only the minority of cells which died in this cycle.

There was no significant correlation between the duration of the cycle time of mother cells and the fate of the progeny produced i.e. both very long and relatively short cell cycle times could be antecedent of cells dying in the 3rd cycle.

The kinetics of cells exposed in G2 to MAM acetate (20 μgcm^{-3})

The analysis of cycle times for 42 cells exposed to MAM acetate at the lower dose of 20 μgcm^{-3}, given one hr before mitotic selection (a G2 treatment) is shown in Table 3.

Table 3. Cell cycle times for synchronous HeLa cells exposed to 20 μgcm^{-3} *MAM acetate during the G2 period immediately before synchronization by mitotic selection**

Cycle	Number of cells observed	Interphase time† (min)	Mitotic time† (min)
1	42	1375 ± 70	59 ± 7
2	62	2179 ± 107	79 ± 9
3	73	1382 ± 39	55 ± 5
4	55	1402 ± 39	61 ± 11

*A cell is included if it completes the cycle; cells dying during interphase or mitosis were excluded.

†Mean ± S.E.M.

By comparing these results with those for cells treated with MAM acetate at 100 μgcm^{-3} in the G1 phase of the cell cycle, it can be seen that a similar disturbance of kinetics has occurred, although less in extent. By the 3rd cycle, both interphase and mitotic times have

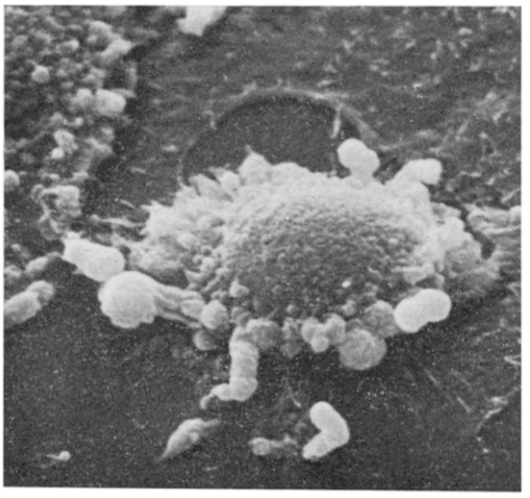


Fig. 2.

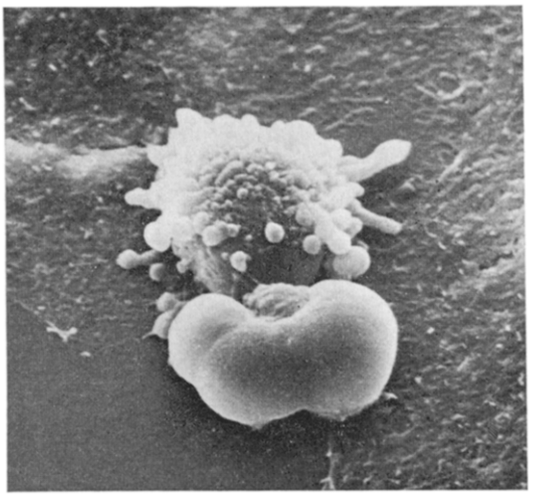


Fig. 3.

Figs. 2 *and* 3. *Scanning electron micrographs illustrating the surface blebs and protrusions which are associated with impending cell death. These cells were observed in synchronous cultures* 50–100 *hr after exposure to* 100 $\mu g cm^{-3}$ *MAM acetate.*

(to face p. 716)

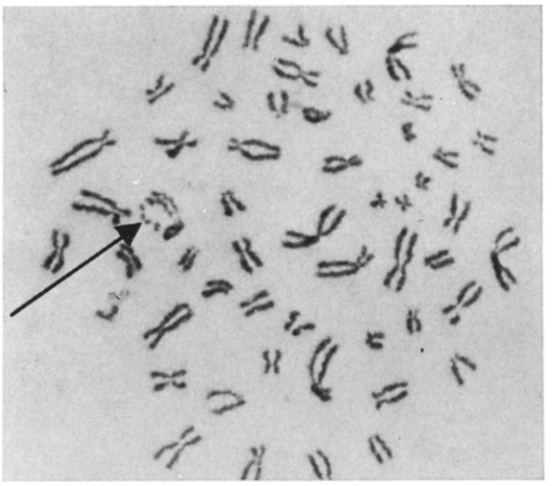


Fig. 6(*a*).



Fig. 6(*b*).

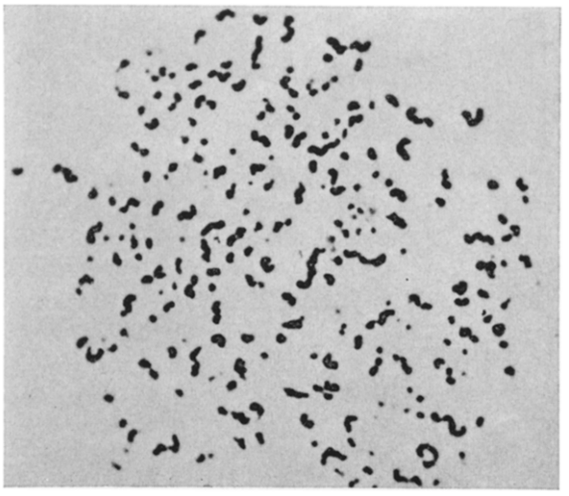


Fig. 6(*c*).

Fig. 6. *Chromosome aberrations detected at the time of the onset of the 2nd mitosis.* (*a*) *localized to a single chromosome.* (*b*) *gaps, breaks and exchanges seen in several chromosomes.* (*c*) *extensive fragmentation,* (*pulverization*) *affecting all chromosomes.*

returned to values compatable with those for untreated cells. All the cells survived the 1st cycle and completed mitosis. Survival in the 2nd or 3rd cycles was higher at this dose than for cells exposed to 100 μgcm^{-3} MAM acetate. This is shown in Fig. 5. In the 2nd cycle, 17% of the cells failed to survive and the majority of deaths were associated with a period of rounding up which lasted several hours before the onset of the terminal sequence. The timing for this type of death was 4019 ± 243 min after mitosis. Four per cent of the cells in the 2nd cycle neither divided nor died during the 6 days of observation.

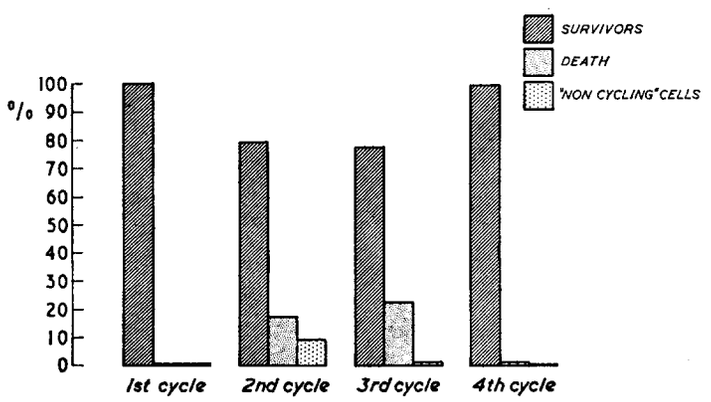


Fig. 5. Distribution of death and survival in each cycle following exposure to MAM acetate (20 μgcm^{-3}, G2 treatment).

In the 3rd cycle, 77% of the cells survived and were seen to complete mitosis. Of the remainder, 21% died, and in the majority of deaths, no prior period of rounding up was seen. The timing for this pattern of death was 679 ± 132 min from mitosis.

The analysis of clonal death

The fate of daughter pairs in the 2nd and 3rd cycles, at both levels of MAM acetate investigated, has been analysed and is shown in Table 4.

The daughter pairs were classified according to whether both cells completed the cycle successfully (both survive), whether one daughter survived and the other died within that cycle (one survives), or whether both cells died within the same cycle (both fail).

These results indicate that at the two levels investigated, those effects of MAM acetate which are sufficient to cause the death of the cell, are not equally expressed by the daughter cells and that losses from within a given clone can occur in both the 2nd and 3rd cycles, leading either to the complete disappearance of the clone or to the selection of a single cell or two cells that will undertake mitosis and give rise to progeny exhibiting normal cell cycle times.

A comparison of the size of clones that had remained in the field of view at the end of the 3rd cycle (Table 5), shows that at the higher level (100 μgcm^{-3} MAM acetate), all the first generation cells have been affected and had received injury sufficient to affect one or more of the progeny. However, at the lower level (20 μgcm^{-3}), 29% of the originally treated cells sustained no lethalities in any of their progeny.

Chromosome analysis

The chromosome spreads showed no difference between the treated cells (100 μgcm^{-3}) and controls until 48 hr after treatment. Thereafter, there was an increasing number of mitotic figures showing chromosomal aberrations, which reached a peak of about 50% incidence, at 80–90 hr. Aberrations could still be found on the 6th day (144 hr) and the 8th day (192 hr) after treatment, but were absent on the 14th day (336 hr).

Table 4. The fate of daughter pairs of treated cells

Cycle	Dose (μgcm^{-3})	Treatment phase	Both survive	One survives	Both fail	"Non-cyling"*
2nd	100	G1	35%	30%	22%	13%
3rd	100	G1	18%	16%	38%	28%
2nd	20	G2	67%	10%	13%	10%
3rd	20	G2	73%	15%	12%	0%

*These pairs contained cells which neither died nor divided by the end of the experiment.

Table 5. Distribution of clones by size (number of viable cells) at end of the 3rd interphase

	0 cells*	1 cell	2 cells	3 cells	4 cells	"Non-cycling†
Control					100%	
100 μgcm^{-3}	44%	10%	22%	0%	0%	24%
20 μgcm^{-3}	17%	8%	8%	17%	29%	21%

*This column represents those treated cells, all of whose progeny died during the course of the experiment.

†Clones that contained "non cycling" cells i.e., their exact fate is not known.

The aberrations were either localized to single chromosomes (Fig. 6a) or were seen in several or all the chromosomes as variable patterns of gaps, breaks and chromatid exchanges (deletions and ring chromosomes) (Fig. 6b). The damaged chromosomes were frequently loosely coiled. The most severe aberrations seen were complete fragmentations (pulverizations) of the chromosomes (Fig. 6c).

The cytological preparations showed that these chromosomal aberrations were associated with the formation of anaphase bridges and deletions in one or several chromosomes, due to the failure of acentric fragments to migrate to the poles of the mitotic spindle.

DISCUSSION

The cell kinetics presented in this investigation are derived from the detailed analysis of small numbers of cells. Although a method now exists for calculating the true cycle time distribution from censored samples [13], this is only applicable to systems in exponential growth with a stable age distribution. The present study is to be regarded as a direct extension of the work undertaken in this laboratory by Van den Berg and Ball [7], who have studied this system biochemically and have obtained supporting evidence from autoradiographic, cytometric and microdensitometric analyses. With microcinematography, it is necessary to film an initially low concentration of cells, otherwise the culture will reach confluence before the behaviour of the progeny can be observed for several generations. The intrinsic limitations of the photographic field is another factor restricting the sample size.

This study has made it reasonably clear that all cells in the system are affected by exposure to MAM acetate but that the effect is minimal during the 1st cell cycle. The cells that do survive and eventually give rise to new colonies, have themselves been subject to a prolongation of interphase in the 2nd cycle.

Such a pattern of delayed expression has also been found to be the response of this cell line to injury by the alkylating agent, MNU [8]. Van den Berg [14] has further reported that at the dose level of 100 μgcm^{-3} MAM acetate, the major abnormality detected in the first cycle is a failure to ligate replicon sized segments of newly synthesized DNA. This effect is apparently corrected within five hr, but following cell division, a failure to ligate replicon sized segments of DNA occurs again in the second cycle and this is associated with a dose dependent inhibition of DNA synthesis.

These observations support the analysis of the timing of the appearance of chromosomal aberrations. Examination of metaphase chromosomes in the first mitosis following treatment failed to reveal the presence of aberrations, but chromosomal aberrations were seen in metaphase spreads prepared at the time of the onset of the 2nd mitosis. Furthermore, the severity of the aberrations seen in a proportion of the mitotic figures supports the concept that the majority of cell death in the second cycle is associated with an arrest in mitosis. Extensive fragmentation (pulverization) is incompatible with cell division and gross chromatid exchanges would lead to major mechanical difficulties of chromosome separation during anaphase.

A similar relationship between mitosis and death has been demonstrated before. Hurwitz and Tolmach [15] observed directly that three quarters of cell death occurring in populations of HeLa cells exposed to 220 kV X-rays was associated with an arrest in metaphase.

The other pattern of cell death observed in this investigation and which is responsible for the majority of cell loss during the 3rd cycle, occurs 10–12 hr after mitosis; that is at a time when survivors would be expected to be synthesising DNA. Although it is naïve to attempt to explain all these cell deaths in relation to the aberrations observed, the probability exists that a proportion of this cell loss in the 3rd cycle is the consequence of genetic deletions and the associated loss of vital information.

The analysis has also revealed the presence, in both the 2nd and 3rd cycles, of a group of cells that are considered as being "non-cycling". These cells remained in an extended interphase and were not seen to die or divide during the

period of observation. Presumably the damage inherited by these cells is not sufficient in itself to cause death directly, but it is extensive enough to delay severely the initiation of those events which prepare the cell for the next mitosis.

By using a technique which preserves the familial relationships of cells, it can be seen that the lethal effects of exposure to MAM acetate, at the two levels investigated, are not exhibited equally by the daughter pairs. The results in Table 4 show that in both the 2nd and 3rd cycles, the situation exists where one member of a pair may die, whilst the other survives. It is to be expected that such a situation would follow the occurrence of chromosome damage, as this would account for a non equal transmission of genetic information, but the finding that this situation also occurs in the 2nd cycle suggests that either there is a non equal transmission of damaged DNA during the 1st mitosis or, more likely, that some sort of modifying factor exerts an effect in the 2nd cycle. In a similar analysis of the effects of exposure to bleomycin in L cells, Katsumata *et al.* [16] have shown that an unequal occurrence of death between daughter cells also occurs at doses permitting 30 % survival. However, they found no evidence of this at the higher level of bleomycin studied (10 % survival) and concluded that the damage induced by the antibiotic at the lower concentration was not great enough to be distributed equally into sister cells.

In general, the experience of using microcinematography has led to the conclusion that this method is useful in resolving complex cell kinetic problems, in which members of a cell population are exhibiting a wide range of individual patterns of behaviour; and where mathematical treatments are inapplicable due to the major perturbations of mitosis itself and the dynamic nature of the parameters of the cell cycle. In such circumstances, it could well prove to be a valuable tool for the study of drug treatment.

REFERENCES

1. G. L. Laquer, E. G. McDaniel and H. Matsumoto, Tumour induction in germ free rats with methylazoxymethanol (MAM) and synthetic MAM acetate. *J. nat. Cancer Inst.* **39,** 355 (1967).
2. M. Spatz, D. W. E. Smith, E. G. McDaniel and G. L. Laquer, Role of intestinal micro-organisms in determining cycasin toxicity. *Proc. Soc. exp. Biol.* **124,** 691 (1967).
3. T. Narisawa and H. Nakano, Carcinoma of the large intestine of rats induced by rectal infusion of methylazoxymethanol. *Gann* **64,** 93 (1973).
4. M. G. Gadbridge, A. Denuzio and M. S. Legator, Cycasin; detection of associated mutagenic activity *in vitro*. *Science* **163,** 689 (1969).
5. H. J. Teas and J. G. Dyson, Mutation in drosophila by methylazoxymethanol; the aglycone of cycasin, *Proc. Soc. exp. Biol.* **125,** 988 (1967).
6. H. Matsumoto and H. H. Higa, Studies on methylazoxymethanol acetate, *Biochem. J.* **98,** 20c (1966).
7. H. W. Van Den Berg and C. R. Ball, The effect of methylazoxymethanol acetate on DNA synthesis and cell proliferation of synchronous HeLa cells. *Mutation Res.* **16,** 381 (1972).
8. 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, *Chemico. biol. Interactions* **3,** 337 (1971).
9. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, Hydrogen ion buffers for biological research. *Biochemistry* **5,** 467 (1966).
10. E. Robbins and P. T. Marcus, Mitotically synchronized mammalian cells; a simple method for obtaining large populations. *Science* **144,** 1152 (1964).
11. Z. H. Price, The micromorphology of zeiotic blebs in cultured human epithelial cells (HEp) cells. *Exp. Cell Res.* **48,** 82 (1967).
12. M. Bessis, Studies on cell agony and death: an attempt at classification, In *Cellular Injury*. (Edited by A. V. S. Reuck and J. Knight) CIBA General Symposium, Little & Brown, Boston (1964).
13. P. Jagers and K. Norrby, A new method of estimating the mean cycle time and the dispersion of cycle times in exponential growth as applied to monolayer cell populations. *Cell. Tiss. Kinet.* **7,** 201 (1974).

14. H. W. Van Den Berg, Alkaline sucrose gradient sedimentation studies of DNA from HeLa S_3 cells exposed to methyl methanesulphonate or methylazoxymethanol acetate. *Biochem. biophys. Acta.* In press (1974).
15. C. Hurwitz and L. J. Tolmach, Time lapse cinemicrographic studies of X-irradiated HeLa S_3 cells. *Biophys. J.* **9,** 607 (1969).
16. T. Katsumata, M. Watanabe, Y. Takabe, T. Terasima and U. Umezawa, Cinematographic analysis of death in synchronously growing mouse cells after exposure to bleomycin. *Gann* **64,** 71 (1973).

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An *In Vitro* Method for Assaying Sensitivity to 2,4-Dinitrochlorobenzene (DNCB) in Man

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Abstract—*There is a need for an* in vitro *method for assaying sensitivity to dinitrochlorobenzene in patients being studied serially who develop severe local reactions to skin testing. Both lymphocyte stimulation and leucocyte migration inhibition tests have been used in attempts to assay sensitivity to DNCB in patients being studied serially. Best results were obtained using the leucocyte migration inhibition test with an aqueous solution of DNCB as antigen. This* in vitro *test was positive in* 23/26 *skin test positive patients and was negative in* 16/18 *unsensitised patients.*

INTRODUCTION

DINITROCHLOROBENZENE (DNCB) is used widely to assess the delayed hypersensitivity skin reactions in patients with suspected immunological impairment. Studies in patients with cancer have shown that response to DNCB correlates with prognosis and that the response of the patient to serial testing reflects the course of the illness [1, 2]. Serial testing also has potential clinical application in the quantitation of immunosuppressive therapy in organ transplantation [3].

The standard DNCB test consists of application to the skin of a sensitising dose of DNCB followed by application of a challenge dose two weeks later. The response is recorded 48–72 hr later. While most patients tolerate the procedure well, occasionally a severe local reaction develops and further tests are contraindicated. It is when serial studies are planned that an *in vitro* method for assessing sensitivity to DNCB is needed.

Both the lymphocyte stimulation test [4] and the leucocyte migration inhibition (LMI) test [5] have been shown to correlate with delayed hypersensitivity skin tests in man. We have studied both of these methods using DNCB dissolved in medium and DNCB conjugated to lymphocytes [6] as antigen. A good correlation with skin tests was obtained using the LMI test, with DNCB dissolved in medium as antigen.

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MATERIAL AND METHODS

Patients

Patients were sensitised to DNCB by the method of Brown *et al.* [7]. In brief, 2000 μg of DNCB dissolved in acetone was applied to the upper arm and allowed to evaporate. Pre-existing sensitivity was excluded by simultaneous application of 100 μg DNCB to the forearm. Fourteen days later, challenge doses of 50 and 100 μg were applied to the forearm. Responses were read at 72 hr. A positive test consisted of induration and erythema at the test site. Skin testing was performed at the time of venepuncture in sensitised patients except where contraindicated because of a previous strong reaction. These latter patients were regarded as skin test positive. Non-sensitised subjects were hospital patients with a variety of illnesses. They were not skin tested and were regarded as skin test negative.

Antigen preparation

The DNCB (1-chloro-2,4-dinitrobenzene, Eastman Kodak, Rochester, N.Y.) solution was prepared by boiling DNCB in water, allowing it to cool and recrystallise. It was then filtered and the slightly yellow solution was stored at room temperature in the dark. The concentration of DNCB was assayed before use on each occasion by measurement of absorption at 330 mμ in a spectrophotometer. The DNCB was then further diluted in medium to give a final concentration of 1·10–1·15 μg/ml.

2,4-dinitrobenzene sulphonic acid sodium salt (DNSB) (Eastman Kodak, Rochester, N.Y.) was dissolved in medium at a concentration of 2 μg/ml.

DNCB-lymphocytes were prepared by the method of Miller and Levis [6]. Washed lymphocytes ($1{\cdot}25 \times 10^6$/ml) were incubated with DNCB dissolved in dimethyl sulphoxide (DMSO) (100 mg/10 ml) for one hr at 37°C. The lymphocytes were washed three times with saline, re-suspended at a concentration of $1{\cdot}25 \times 10^6$/ml and lyophilised in 1 ml aliquots. Control lymphocytes were prepared by incubation in DMSO alone and treated as above.

DNSB-lymphocytes were prepared by incubation of lymphocytes with a solution of DNSB in medium (100 mg/10 ml) and washed and lyophilised as above. Control lymphocytes were prepared by lyophilising lymphocytes incubated in medium alone.

Leucocyte migration inhibition test

Leucocytes were separated from 15 ml of heparinised blood (20 units of preservative-free heparin/ml) by sedimentation over hypaque-methylcellulose at 1 **g**. The leucocytes were washed twice in saline, suspended in McCoy's 5A medium + 15 % foetal calf serum (FCS) and taken up in micro-haematocrit tubes (Gelman Hawksley Ltd., Lancing). The capillaries were sealed with Cristaseal (Gelman Hawksley Ltd., Lancing), centrifuged at 400 **g** for five min and cut at the cell-medium interface. The capillary containing cells was placed in a well of a migration plate (Sterilin Ltd., Richmond) containing medium alone (McCoy's 5A medium + 15 % FCS) or medium plus antigen. The cells were allowed to migrate overnight at 37°C and the migration area measured by projection and planimetry. Tests were performed in quadruplicate. Results were expressed as:

$$\frac{\text{area of migration of test}}{\text{area of migration of control}} \times 100$$

Migration in the test wells of less than 80 % of control values was considered a positive result.

Lymphocyte stimulation test

Lymphocytes were obtained from 15 ml of blood (20 units preservative-free heparin/ml) using ficoll-hypaque [9]. The lymphocytes were washed three times and re-suspended in McCoy's 5A medium, containing 15 % FCS and 14 mM Hepes, at a concentration of $1{\cdot}25 \times 10^6$/ml. The cells were dispensed into the wells of a microculture plate (Linbro IS-FB-96, Biocult Ltd., Paisley) in 0·2 ml aliquots ($2{\cdot}5 \times 10^5$ cells/well). The antigen was added in a volume of 0·05 ml and the plates incubated for seven days at 37°C in an atmosphere of 5 % CO_2. Four hr before harvesting, 0·05 ml of 10 μCi/ml of tritiated thymidine (specific activity (20 Ci/mM, Radiochemical Centre, Amersham) was added and the plates re-incubated. For harvesting, the cells were washed from individual wells into tubes containing cold saline, washed on to glass fibre filters (GF/C, Whatman's Ltd., Maidstone) and then washed with 20 ml cold 5 % trichloracetic acid, followed by 15 ml methanol. The filter paper was placed in a scintillation vial and allowed to dry. When dry, 0·2 ml Protosol (New England Nuclear Ltd., Reading) was added and allowed to digest the precipitate on the filter for two hr. Scintillation fluid (5g PPO, 0·5 g dimethyl POPOP, 1000 ml toluene) was added and the samples were counted in an LKB-Wallac Scintillation Counter. Results were expressed as disintegrations per min. The stimulation index,

$$\frac{\text{DPM stimulated cultures}}{\text{DPM unstimulated cultures}}$$

was calculated. A stimulation index of >3 was considered a positive test.

RESULTS

Preliminary studies indicated that the concentration of the aqueous DNCB varied considerably depending on the temperature at which it was stored. Best stability was obtained at room temperature (20°C). Titration of the DNCB-medium indicated that concentrations of DNCB of less than 1·05 μg/ml were not active, whereas concentrations of 1.8 μg/ml were toxic to some control lymphocytes. A concentration of 1·1 μg/ml was considered optimal and was used in all tests with aqueous DNCB. In a similar titration of DNSB, no non-specific toxicity was found up to a concentration of 2 μg/ml.

Lymphocyte stimulation tests: (Table 1)

No stimulation was found using DNCB-lymphocytes or DNSB-lymphocytes up to an antigen concentration of $2{\cdot}5 \times 10^5$ DNCB-lymphocytes/ml i.e. an antigen to cell ratio of 1:4. Similar results were obtained after either five or seven days incubation before harvesting. Two out of sixteen tests were positive using DNSB in medium at a concentration of 2 μg/ml. Both of these patients were skin test positive. However, lymphocytes from seven other skin test positive patients failed to react.

Table 1. Lymphocyte stimulation tests

Antigen	Concn	Results	
		*	†
DNCB-lymphocytes	$2{\cdot}5 \times 10^5$/ml	0	22
DNCB	1·1 μg/ml	0	11
DNSB-lymphocytes	$2{\cdot}5 \times 10^5$/ml	0	12
DNSB	2 μg/ml	2	14

Final lymphocyte concn 1×10^6/ml.
*=Stimulation (Stimulation index >3).
†=No stimulation.

Leucocyte migration inhibition tests (Table 2)

Neither DNSB or DNSB-lymphocytes were reactive as antigen. DNCB-lymphocytes (50,000 cells/well) gave the correct result in 4/6 cases but failed to detect 2/5 skin test positive patients. Best results were obtained using DNCB in medium. Using a concentration of 1·1 μg/ml, the test correlated with skin testing in 39/44 patients. Two out of eighteen unsensitised patients were positive on *in vitro* testing while 3/26 skin test positive patients were negative using the LMI test.

DISCUSSION

There is a need for an *in vitro* method of assaying response to DNCB particularly where serial follow-up testing is planned in patients who develop significant skin reactions. There have been a number of reports of *in vitro* methods for assaying sensitivity to DNCB in guinea pigs. Geczy and Baumgarten [10] conjugated living lymphocytes by incubation with dinitrofluorobenzene (DNFB) solution. They found that these conjugated lymphocytes acted as a specific antigen in a lymphocyte stimulation test using peripheral lymphocytes from sensitised guinea pigs. They failed to obtain stimulation using erythrocytes or serum proteins conjugated with DNFB or using heat-killed DNFB-lymphocytes. Friend and Lane [11] used a macrophage migration inhibition test with peritoneal macrophages from sensitised guinea pigs and obtained specific inhibition using a dinitrophenol (DNP)-serum protein conjugate. Nishioka and Amos [12] obtained specific inhibition using epidermal microsomes isolated from guinea pig skin which had been painted with DNCB. Instead of using macrophages from sensitised animals, they studied the release of migration inhibition factor (M.I.F.) from lymphocytes of sensitised animals.

Miller and Levis [6] have adapted the techniques of Geczy and Baumgarten [10] and have studied lymphocyte stimulation in humans who were being treated for multiple basal cell carcinomas using DNCB. They obtained best results using DNCB-conjugated lymphocytes as antigen, the lymphocytes being killed during the conjugation process. All of their subjects had been sensitised with repeated and high doses of DNCB and were tested when sensitivity developed.

This is different from the situation in a

Table 2. Leucocyte migration tests

Antigen	Concn	Migration Inhibition	
		*	†
DNCB-Lymphocytes	50,000 cells/well	3 (3+)‡	3 (2+, 1−)
DNSB-Lymphocytes	50,000 cells/well	1 (+)	5 (3+, 2−)
DNSB	2 μg/ml	0	5 (5+)
DNCB	1·11 μg/ml	25 (23+, 2−)§	19 (3+, 16−)§

*Inhibition (Migration <80%).
†No inhibition.
‡Figures in brackets are the skin test results.
§X^2 test, $P < 0{\cdot}4 \times 10^6$.

follow-up study where the patient may have been sensitised several years previously and only skin tested every six months. This might explain why lymphocytes from our patients were not stimulated using DNCB-lymphocytes as antigen.

The LMI test has been shown previously to correlate well with skin testing using a number of antigens [5]. Using DNCB in medium as antigen it has given a good correlation with skin testing in this study. It is uncertain how the DNCB solution is acting as an antigen in this test. DNCB is believed to act as a hapten and to conjugate to a carrier protein in order to become immunogenic. This carrier protein has not been identified. The previously reported studies [6, 10–12] have each produced evidence for a different carrier. It is possible that in our system, the DNCB is binding with the patient's lymphocytes in the test chamber, forming an immune complex as suggested by Geczy and Baumgarten [10].

There are a number of technical problems associated with the use of DNCB in aqueous solution. It is only sparingly soluble and the level of solubility changes with temperature. Because of this, we found it necessary to assay the concentration each day before use. The range over which it is active is very narrow and has to be ascertained carefully.

Because of these technical problems, we feel that in routine testing it is quicker and simpler to use the standard skin test procedure. However, where a patient has a severe local skin reaction and further tests are contraindicated, the LMI test using DNCB in aqueous solution offers a method for continuing follow-up studies.

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REFERENCES

1. F. R. Eilber and D. L. Morton, Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer (Philad.)* **25,** 362 (1970).
2. P. M. Bolton, S. L. James, J. M. Davidson and L. E. Hughes, Diagnostic and prognostic significance of delayed hypersensitivity skin testing in patients with malignant neoplasia. *Brit. J. Cancer* **28,** 80 (1973).
3. W. L. Joseph, F. Melewicz and D. L. Morton, Delayed cutaneous hypersensitivity to dinitrochlorobenzene as a method of quantitating immunosuppressive therapy for organ transplantation. *Surgery* **69,** 847 (1971).
4. J. J. Oppenheim, Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. *Fedn. Proc. Fedn. Am. Socs. exp. Biol.* **27,** 21 (1968).
5. M. Søborg and G. Bendixen, Human lymphocyte migration as a parameter of hypersensitivity. *Acta med. scand.* **181,** 247 (1967).
6. A. E. Miller and W. R. Levis, Lymphocyte transformation during dinitrochlorobenzene contact sensitisation. *J. clin. Invest.* **52,** 1925 (1973).
7. R. S. Brown, H. A. Haynes, H. T. Foley, H. A. Godwin, C. W. Berard and P. P. Carbone, Hodgkin's disease. Immunological, clinical and histological features of 50 untreated patients. *Ann. intern. Med.* **67,** 291 (1967).
8. A. Böyum, Separation of white blood cells. *Nature (Lond.)* **204,** 793 (1964).
9. A. Böyum, Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest.* Suppl. **21, 97,** 77 (1968).
10. A. F. Geczy and A. Baumgarten, Lymphocyte transformation in contact sensitivity. *Immunology* **19,** 189 (1970).
11. J. V. Friend and M. Lane, *In vitro* studies of contact sensitivity. The effect of the haptens, 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrofluorobenzene (DNFB) and of hapten–protein conjugates on the migration of guinea-pig peritoneal exudate cells. *Immunology* **25,** 869 (1973).
12. K. Nishioka and H. E. Amos, Contact sensitivity *in vitro*. The production of macrophage inhibition factors from DNCB sensitised lymphocytes by subcellular organelles obtained from DNCB epidermal tissue. *Immunology* **25,** 423 (1973).

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Radio-Immunoassay of Casein in the Serum of Normal Subjects and of Patients with Various Malignancies*

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Abstract—*This paper describes the establishment and use of a radioimmunoassay of casein in the serum of normal subjects and patients with cancer. The casein used for labelling, antibody production and as a reference preparation was extracted from human milk. Labelling with ^{125}I using the chloramine T method. The radioimmunoassay was carried out either by the double antibody or by the double antibody solid phase technique. Casein is not normally detectable in the serum of men or non-pregnant women; on the other hand, all samples from lactating women contain the material.*

In patients with breast cancer prior to any therapy, casein is detectable in 72%. *After treatment when there has been local recurrence or distant metastases,* 80% *of patients are positive. We have also found this substance in* 50% *of patients with gastro-intestinal carcinoma and in* 7 *patients with lung cancer.*

INTRODUCTION

CASEIN is one of the protein constituents of milk. Its secretion is under the control of various hormones, particularly of prolactin [1, 2]. Whether this normal and specific constituent of the exocrine secretion of the mammary gland can also be found in blood is, at present, unknown. It thus was of interest to us to establish a radioimmunoassay of human casein and to seek its presence in serum in physiological circumstances when the breast is active, i.e. during pregnancy and lactation.

We also sought its presence in situations not associated with lactation in men and women during the menstrual cycle and after the menopause.

The presence of casein was particularly carefully sought in the blood of women with breast cancer.

It could in fact be postulated that an exogenous secretion might persist in these neoplasms because histological preparations of certain tumours of the breast show the presence of milk secretion in the mammary ducts and the growth of the tumour maintains a greater or lesser degree of dependence on the mammotrophic hormones. If this phosphoprotein could be found in the blood of a patient suffering from breast cancer, the measurement of casein might facilitate early diagnosis as well as the ability to follow the course of treated breast tumours. It could also serve the same purpose in patients suffering from cancers of other sites if an ectopic exocrine secretion of neoplastic origin could be demonstrated.

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MATERIAL AND METHODS

1. *Preparation of human casein*

Casein was isolated from human milk using a method of preparation at constant pH [3, 4]. We stopped at the stage termed "first cycle soluble casein" which in fact is a mixture of I, II and K casein. After 48 hr dialysis at 4°C against distilled water, this preparation was lyophylized and kept at − 20°C.

Using the methods of Groves and Gordon [5], highly purified II and K casein fractions were obtained. These fractions were used as tracers.

The purity of these preparations of II and K casein were established by the homogeneity after filtration on Sephadex G 200 and chromatography on DEAE cellulose. Furthermore, using the immunoelectrophoresis and agar gel diffusion techniques, the K preparation gave a simple line of precipitation with the anti casein serum. The line of precipitation did not disappear when the anti casein serum was pretreated with normal male and female serum. Moreover, the anti human protein serum did not react with these two casein fractions.

This line of precipitation was situated in the zone where β globulins normally migrate in a more cathodal position than lactalbumin and lactoferrin.

2. *Anti-serum production*

Four rabbits were immunized by the intradermal route [6]. Two hundred and fifty μg were used for each immunization. For each rabbit an intial booster was given 2 months later using the same dose and following the same procedure. After the first booster, 2 rabbits died, probably due to anaphylactic shock. The other two animals were given a second booster 2 months later. Six weeks later the properties of the antisera made them suitable for use in a radioimmunoassay of casein—at a dilution of 1 in 30,000, 50 % of 1 ng of labelled casein was bound to the antibody.

The anti casein serum was treated with normal human serum in a ration of 9:1 in order to neutralise possible antibodies directed against serum proteins.

As previously described, a single line of precipitation was observed in the migration zone of β globulins where anti casein serum reacted with K casein preparation in immunoelectrophoresis. The same line of precipitation was obtained with milk but is not found with hydrosoluble fraction of sperm and saliva.

3. *Labelling of casein*

Casein was labelled by modification of the chloramine T method [7]. The reaction was carried out in a labelling tube containing 25 μl of a sodium ^{125}I (1 mCi, I.R.E., Belgium) in a phosphate buffer pH 7·5 0·05 M. Subsequently 25 μl of 0·5 M phosphate buffer pH 7·5 were added to buffer the medium. The remaining reagents were dissolved in phosphate buffer 0·05 M pH 7·5. Five μg of casein in 25 μl and 25 μg of chloramine T in 25 μl were added successively; oxidation was allowed for 30 sec and the action was then stopped with 120 μg of metabisulfite in 100 μl. Radioactive iodine was separated from labelled protein by gel filtration on a column of Sephadex G 50 medium (0·9 × 15 cm) equilibrated in phosphate buffer 0·05 M pH 7·5 and presaturated with bovine albumin. The labelled casein was then submitted to a second purification on a column of Sephadex G 200 (0·9 × 40 cm) equilibrated in phosphate buffer 0·05 M pH 7·5 and 0·1 % bovine albumin. The mean specific activity was between 86 and 132 μCi/μg.

4. *Assay method used*

Alternatively, the double antibody method [9] or the double antibody solid phase method (DASP) [8–10] was used.

The incubation medium consisted of phosphate buffer 0·05 M pH 7·5 containing 0·05 % NaN_3 and 0·5 % bovine albumin. Total incubation volume was 0·4 ml containing 1 ng of labelled casein, anticasein antiserum diluted 1 in 30,000 and either a standard preparation of casein or 0·1 ml of the unknown serum. To all tubes used for the establishment of the standard curve, 0·1 ml of a pool of normal male serum was added so that the concentration of human serum protein would be the same in all the tubes. With the double antibody solid phase method, incubation was 20 hr at room temperature. One half ml of a suspension of the anti-γ-globulin immunadsorbent was added this consisted of cellulose to which rabbit anti-γ-globulin had been covalently linked [11]. The immunoadsorbent suspension was diluted in incubation buffer containing 5 ml per litre of Tween 20.

The mixture was shaken by slow rotation for 4 hr at room temperature and the immunoadsorbent was subsequently separated by centrifugation, washed and counted in a solid gamma scintillation counter.

On the other hand, when double antibody was used incubation was for 24 hr at room temperature, 0·1 ml of normal rabbit serum (1/100) and 0·1 ml of anti rabbit γ globulin serum (1/10) were added. Precipitation was allowed to proceed for 16 hr and the precipitate was then separated by centrifugation, washed and counted.

To assess the specificity, human milk (0·1 ml diluted 1/400 to 1/1000), saliva (1–10 μl) and hydrosoluble fraction of human sperm (50–1000 ng) were also allowed to react in this system.

5. *Patients studied*

The possible effect of EDTA ($1{\cdot}2 \times 10^{-2}$ mole/liter) and Trasylol (100 IU/tube of incubation) has been studied in the tested sera†. Casein was sought in the following normal sera in: 27 pregnant women at different stages of pregnancy, 11 lactating women, and 74 normal men and 36 normal women of various ages.

Casein was measured in the serum of patients suffering with breast cancer.

Seven patients had benign breast tumours (fibro-adenoma, lipomatous cyst) diagnosed by biopsy and histological analysis independently of the casein measurements.

Eleven patients with the histological diagnosis of breast cancer. Casein was measured prior to surgical treatment, radiotherapy or treatment with cytostatic drugs.

Forty-one patients with breast cancer at Stage 1 (T_1, T_2, N_o, N_a) were treated surgically (Halsted operation or simple mastetomy with the removal of regional lymph nodes) and treated post-operatively by irradiation. There was no evidence of metastases on clinical examination, pre-operative radiology and histological analysis of the material removed surgically.

Sixty-one patients with breast cancer had been treated surgically and irradiated after this treatment; there was evidence of metastases in the axillary lymph nodes and they therefore belonged to Stage II (T_1, T_2, N_o).

Twenty-six patients with breast cancer were studied who had evidence at the time of measurement of local recurrence and/or the presence of new metastases despite surgical treatment, radiotherapy and sometime chemotherapy.

We also sought the presence of casein in the serum of 14 patients with neoplasma of the gastrointestinal tract and 7 with carcinoma of the lung.

RESULTS

1. *Labelling*

In our preliminary experiments, the mixture of I, II and K casein was labelled resulting in an unstable tracer. The use of casein II or K for labelling gave more stable tracers. On Sephadex G 200 homogeneous peak retaining full immunoreactivity with antibody was eluted just after the void volume of the column (mol wt $\pm$ 150,000). This elution profile indicated that the labelled II or K casein exist in polymeric form in our experimental conditions. After the second purification, the labelled II or K casein could be stored for at least 8 days without any damage. Furthermore, after 8 days of incubation with serum, there is no evidence of incubation damage of these two tracers.

The use of the mixture of I, II and K casein or the use of pure II or K casein as tracers resulted in standard curves which were very similar provided that the same standard preparation was used.

2. *Characteristics of the assay method*

The smallest detectable dose (defined as the lowest concentration of unlabelled antigen which when added to a solution containing only labelled antigen and antibody, results in significant change in the response variable i.e. 2 S.D.) was 10 ng [12, 13]. Precision as measured by the coefficient of variation within the assay was 8 %.

Human breast milk was able to displace labelled casein bound to antibody whilst cow's milk under the same conditions was not.

On the other hand, the hydrosoluble fraction of sperm did not decrease the radioactivity bound to antibody. The saliva produced some degree of inhibition which was observed in all the other radioimmunoassay systems used to assay follicle stimulating hormone (FSH), luteinising hormone (LH), thyro-stimulating hormone (TSH), α feto protein (AFP) and carcino-embryonic antigen (CEA).

The addition of EDTA ($1{\cdot}2 \times 10^{-2}$ mole/liter) and Trasylol (100 IU/tube) was without effect on the casein level found in the sera.

3. *Detection of casein in serum*

Casein was found in all the sera of lactating women and in none of the pregnant women. Four of 74 men (5 %) and 3 of 36 normal women who were neither pregnant nor lactating (3 %) had detectable levels of casein (Fig. 1).

Only 1 of 7 patients with benign breast lesion had detectable concentration of casein: 350 ng/1 ml of serum.

Eight of 11 patients with breast cancer prior to any treatment had easily detectable levels in their serum—(72 %). Amongst the 41 treated breast cancer patients at Stage I, 17 had significant casein levels (41 %). The levels found were usually moderate and the mean value of the positive cases is 500 ± 160 (S.E.M.) (Fig. 2, Table 1).

In patients in whom casein was detected, the interval between treatment and casein measurement varied from 6 months to 5 years.

†We thank Dr. H. Becker (Erlangen, Germany) and Dr. U. Gaspard (Liège) who kindly provided us with some of these sera.

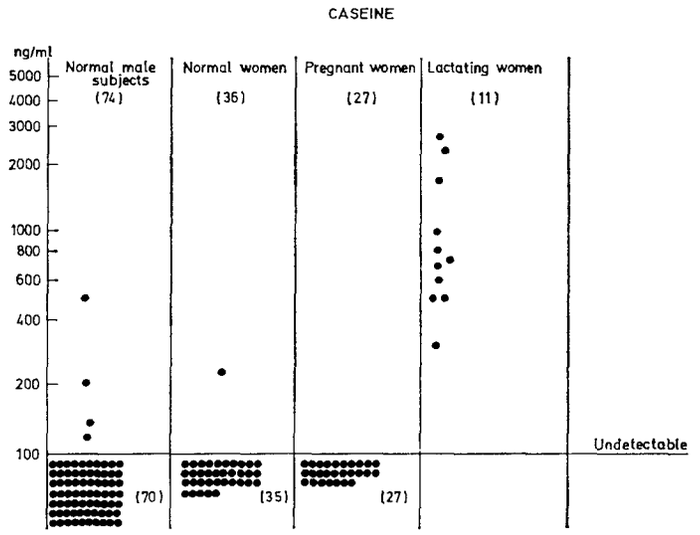


Fig. 1. *Casein levels in the serum of normal men and women, of pregnant women and those who are lactating.*

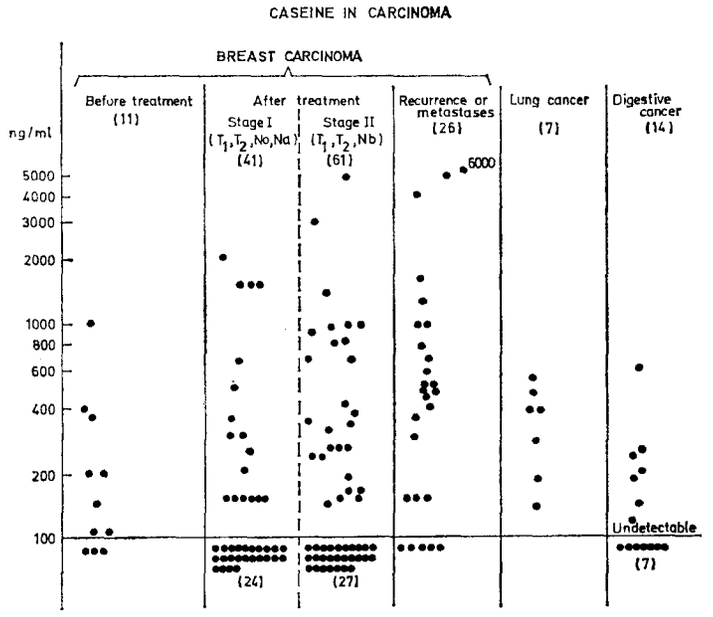


Fig 2. *Levels of casein in the serum of patients with various malignancies.*

Table 1.

	Breast carcinoma				Lung carcinoma	Digestive carcinoma
	Before treatment	After treatment at Stage I	Stage II	With local recurrences and metastases		
Total number	11	41	61	26	7	14
Number of cases with detectable casein	8	17	27	21	7	7
% positive	72	41	50	80	100	50
Mean values of positive cases ± S.E.M. (ng/ml)	283 ± 105	588 ± 160	781 ± 200	1235 ± 360	342 ± 57	235 ± 60

Among the 61 patients with stage II cancers treated surgically and then with post-operative radiotherapy, 34 had detectable casein levels in their blood; the levels found were sometimes very high (Fig. 2).

Casein was found in large quantities in the serum of 21 of 26 patients presenting with a local recurrence or with distant metastases from their tumour. In these patients, the levels were usually markedly elevated and the mean value of positive cases is 1235 μg/ml $\pm$ 360 (S.E.M.) (Fig. 2, Table 1).

In 7 of 14 patients with gastrointestinal neoplasms, casein was found at levels between 120 and 660 ng/1 ml.

Casein was found in the serum of 7 patients with carcinoma of the lung.

DISCUSSION

The radioimmunoassay of casein was carried out using the double antibody solid phase method and double antibody system indifferently.

Contrary to our first experiments using a casein mixture as tracer, labelled K casein or labelled II casein was stable and may be stored or incubated for at least 8 days with no evidence of damage. Thus, there was no decrease in the immunoreactivity and no increase of non-specific adsorption of protein precipitates or on solid phase.

The specific activity ranged between 86 to 132 μCi/μg. If a mol. wt of 25,000 to 30,000 is assumed for the casein monomer [5], approximately 1 atom ^{125}I was bound per molecule.

The specificity of the assay can be established by immunological and radioimmunological arguments. The substance, extracted from milk was not a component of serum or sperm or saliva as determined by immunoelectrophoresis. Furthermore, the zone of migration was more cathodal than lactoferrin and lactalbumin.

By radioimmunoassay, the decrease of the radioactivity was observed with the reference preparation of casein, diluted milk and serum of lactating women whereas there was no inhibition with hydrosoluble fraction of sperm and serum of normal male or female subjects.

Concerning the mild inhibition of untreated saliva in radioimmunoassay of human casein, it was also observed in other radioimmunoassays of hormones (FSH, LH and TSH) and antigens related to cancer (AFP and CEA). This inhibition was not parallel to the reference preparations curves. We think that this effect is non specific since no specific cross-reaction was observed by immunoelectrophoresis. On the other hand, it is well known that mucin present in saliva easily forms insoluble complexes, called "mucin clots" with all sorts of proteins [17] and these could interfere with the first and second immunological reaction.

The characteristics of the radioimmunoassay for casein were very satisfactory as indicted by the smallest detectable amount, the precision and the reproducibility as measured by the coefficient of variation within the assay.

Casein was not usually detectable in the serum of men or of normal women who were not lactating. In contrast, in benign breast lesions this phosphoprotein was found only rarely.

Furthermore, in patients with treated cancer the percentages of patients in whom casein was detectable increased with advancement of the stage of the cancer prior to therapy (Stage I: 41 % and Stage II: 50 %) and with the appearance of local recurrence or distant metastases: 80 %.

Thus, it seems to us that the measurement of serum casein can provide a diagnostic element and a means of follow-up treatment of malignant breast lesions.

The presence of casein in the serum of 41 % of the patients with breast cancer treated at Stage I without any clinical evidence of recurrence could suggest the presence of neoplastic cells pointing to a recurrence of the neoplastic process but still at a sub-clinical stage.

Clinical and biological observations will allow us to determine the significance of detecting casein in patients with cancer. Up to now, we have been unable to establish any correlation between the presence of casein and the histological nature of the tumour; the phosphoprotein can be found with equal frequency in solid carcinoids in scirrhous cancers, in adenocarcinomas and in cancer of the mammary ducts. The presence of casein in serum is not characteristic only of breast cancer; we have in fact found this substance in 50 % of patients with gastrointestinal cancer and in 7 patients with pulmonary neoplasms.

It thus appears that there is ectopic exocrine secretion in addition to ectopic endocrine secretion that has been known for a number of years [14, 15].

Lactoferrin, another protein substance present in milk, has already been studied in the serum of patients with breast cancer [16]. Unfortunately, this substance is present in sperm and in white blood cells which has eliminated all specificity from its findings in serum.

A radioimmunoassay for human casein has

been established. The first step was to extract casein from human milk and to produce specific antibodies; the radioimmunoassay itself is carried out at 18°C using the double antibody or the double antibody solid phase method of separation.

Casein is detectable under normal circumstances only in lactating women. It is not usually detectable in men or in women who are not lactating. In patients with breast cancer casein is detectable in 72% prior to any treatment. In patients with metastases and local recurrence despite treatment, it can be found in 80% of sera. In the follow-up of treatment of breast cancer in Stage I and in Stage II, casein can be found in 41 and 50% of patients respectively. The significance of casein in these patients who are apparently in remission is not yet clear.

Casein thus appears useful as a specific antigen (Tumour marker), the measurements of which may be helpful in the diagnosis and follow-up treatment of cancer.

Detection of casein in cancers other than those of the breast suggests that there may be an ectopic exocrine secretion of this antigen.

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REFERENCES

1. R. W. Turkington, Measurement of prolactin activity in human serum by the induction of specific milk proteins in mammary gland *in vitro*. *J. clin. Endocr.* **33,** 210 (1971).
2. M. Little, The effect of prolactin and growth hormone preparations on the casein production in mouse mammary gland tissue explants. *Acta endocr. (Kbh) Suppl.* **159,** 41 (1972).
3. P. H. von Hippel and D. F. Waugh, Casein: Monomers and Polymers *J. Amer. chem. Soc.* **77,** 4311 (1955).
4. D. F. Waugh and P. H. von Hippel, K-casein and the stabilisation of casein micelles. *J. Amer. chem. Soc.* **78,** 3576 (1956).
5. M. L. Groves and W. G. Gordon, The major component of human casein: A protein phosphorylated at different levels. *Arch. Biochem.* **140,** 47 (1970).
6. J. Vaitukaitis, J. B. Robbins, E. Neischlag and G. T. Ross, A method for producing specific anti-sera with small doses of immunogen. *J. clin. Endocr.* **33,** 988 (1971).
7. W. M. Hunter and F. C. Greenwod, Preparation of iodine 131 labelled human growth hormone of high specificity activity. *Nature (Lond.)* **194,** 495 (1962).
8. A. R. Midgley, R. W. Rebar and G. D. Niswender, Radioimmunoassay employing double antibody technique. *Acta endocr. (Kbh.) Suppl.* **142,** 247 (1969).
9. F. C. Den Hollander and A. H. Schurso, In *Radioimmunoassay Methods.* (Edited by K. E. Kirkham and W. M. Hunter), p. 419. Churchill Livingstone, Edinburgh. (1971).
10. A. M. Reuter, J. C. Hendrick and P. Franchimont, Mise au point d'un dosage radioimmunologique rapide des gonadotrophines. *Ann. Biol. clin.* **31,** 479 (1973).
11. L. Wide, Radioimmunoassay employing immunosorbents. *Acta endocr. (Kbh.) Suppl.* **142,** 207 (1969),
12. H. Feldman and D. Robdard, Mathematical theory of radioimmoassay, In *Principles of Competitive Binding Assays.* Edited by W. D. Odell and W. H. Daughaday, p. 158. Lippincott, Philadelphia (1971).
13. A. R. Midgley, G. D. Niswender and R. W. Rebar, Principles for the assessment of reliability of radioimmunoassay method (Precision accuracy sensitivity and specificity). *Acta endocr. (Kbh.) Suppl.* **142,** 163 (1969).
14. B. F. Bower and G. S. Gordan, Hormonal effects of nonendocrine tumours. *Ann. Rev. Med.* **16,** 83 (1965).
15. M. B. Lipsett, Hormonal syndromes associated with cancer. *Cancer Res.* **25,** 1068 (1965).
16. P. H. Rümke, D. Bisser, H. G. Kwa and A. A. M. Hart, Radioimmunoassay of lactoferrine in blood plasma of breast cancer patients, lactating and normal women, prevention of false high levels caused by leakage from neutrophile leucocytes *in vitro*. *Folia med. neerl.* **14,** 156 (1971).
17. H. E. Schultze and J. F. Heremans, In *Molecular Biology of Human Proteins* Vol. I, p. 769. Elsevier, Amsterdam (1966).

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Therapeutic Effects of an Aromatic Retinoic Acid Analog on Chemically Induced Skin Papillomas and Carcinomas of Mice*

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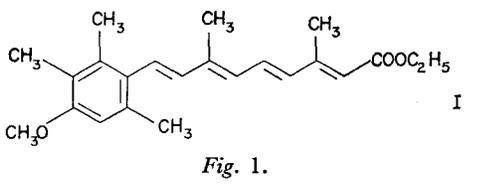
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Abstract—*Retinoic acid has been shown to exert a prophylactic and a therapeutic effect on chemically induced premalignant and malignant epithelial lesions. The development of hypervitaminosis A is the dose limiting factor in its experimental and clinical use. The biological properties of the aromatic retinoic acid analog: ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (= I) were examined. The systemic administration of this compound leads to a marked regression of carcinogen induced established skin papillomas and carcinomas of mice. A method is described for screening of the dissociation between papilloma regression and hypervitaminosis A production. I possesses a therapeutic ratio which is ten times more favorable than that of retinoic acid. The investigation has proven that the antipapilloma effect is not strictly linked with hypervitaminosis A. This raises hopes for finding compounds with an even better therapeutic ratio, useful in the experimental and clinical prophylaxis and therapy of premalignant and malignant epithelial lesions. The mechanism of action is discussed.*

INTRODUCTION

In previous experiments it has been demonstrated that the systemic administration of retinoic acid exerts a prophylactic and a therapeutic effect on skin papillomas and carcinomas of mice induced by 7,12-dimethylbenz(a)anthracene and croton oil [1–3]. Precancerous conditions in man like actinic keratoses of the skin, leukoplakias of the mouth, tongue and larynx as well as papillomas of the urinary bladder responded to a certain degree to a topical or oral treatment with retinoic acid [4–6]. In man the so-called hypervitaminosis A symptoms appear as side effects in the form of headache and lesions of the skin and mucous membranes. These manifestations pose limits to higher dosages. Therefore, derivatives of retinoic acid exhibiting greater antitumor activity and less toxicity respectively were sought.

In animal experiments, the aromatic analog ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (= I) (Fig. 1) showed in comparison with retinoic



I

Fig. 1.

acid a particularly good therapeutic ratio, i.e. a more favorable margin between the effective antitumor dose and the hypervitaminosis A

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*In honour of Prof. Dr. H. Bretschneider, Innsbruck, on the occasion of his 70th birthday.

producing dose. This preparation, therefore, was also selected for clinical trials. Its data of animal experimentation are as follows.

MATERIAL AND METHODS

Female Swiss albino mice of the random bred Füllinsdorf strain weighed 20–22 g at the beginning of the experiment. Controls and test animals received Nafag 199 pellets containing 250 international units of vitamin A per kg of food.

Chemical compounds

1. Retinoic acid = all-trans-β-retinoic acid = vitamin A acid, crystalline powder.
2. Ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate(= I), crystalline powder, synthesized in the Roche laboratories by RUEGG and RYSER, Belgian Patent Application No. 142589, March 29, 1974.

Skin papillomas

Induction of skin papillomas. 7,12-dimethylbenz-(a)anthracene (DMBA) was applied twice to the shaved dorsal skin of mice with an interval of 14 days. One hundred and fifty mcg DMBA, dissolved in 0·2 ml acetone were painted on each mouse on a skin surface of approximately 5 cm^2. After 3 weeks the croton oil treatment was started. One-half mg croton oil, dissolved in 0·2 ml acetone, was applied to the skin of the animals twice a week for 3–8 months. Within this period, most of the animals had developed multiple papillomas, mostly in numbers of 4–10. The therapeutical study was started when the papillomas had reached an average dia of 3 mm.

Therapy of skin papillomas. I was administered i.p. by injection or orally by means of a stomach tube either daily 5 days per week or once weekly for two weeks (days 1 and 8) to groups of4 mice. I as well as retinoic acid was suspended in arachis oil. The doses administered were 12·5–400 mg/kg once weekly or 5–40 mg/kg daily. Twenty mice served as controls and received the vehicle alone. The fluid volumes were 0·04–0·08 ml per 20 g body weight.

Evaluation. The sum of papilloma diameters was determined for each mouse and the average value calculated for each group. These measurements were made at the commencement of therapy (day 0) as well as two weeks (day 14) after the first medication. The increase or decrease of the average sum of the papilloma diameters per animal has been expressed as a percentage of the initial value (day 0).

Skin carcinomas

Induction of skin carcinomas. The above-described method to produce skin papillomas was also used for the induction of skin carcinomas. Whereas skin papillomas appeared mostly 3–8 months after the first application of DMBA, carcinomas usually did not develop until after 5–12 months. The carcinomas rarely emerged primarily as such, but mostly by malignant degeneration of papillomas. In contrast to the papillomas, the carcinomas possessed a broad basis and they infiltrated the subcutaneous tissues. On the surface they were mostly ulcerated in part and grew very rapidly.

Therapy of skin carcinomas. Eleven animals bearing a single carcinoma served as controls whereby the spontaneous growth was observed for a 14-days' test period. The rapid growth of the tumors was reflected in an increase in their length, width and height. Eleven animals with a single carcinoma were treated once a week orally with 400 mg/kg of I. The above described oily suspension was used here too and administered on the first and eighth days of the test by means of a stomach tube. The controls received arachis oil alone.

Evaluation. The approximate volumes of the carcinomas were determined as the product of length × width × height at the beginning (day 0) and at the end (day 14) of the experiment. The increase or decrease was expressed as a percentage of the value on day 0.

Hypervitaminosis A

The so-called hypervitaminosis A symptoms which are manifest as weight loss, redness and scaling of the skin, hair loss, changes in the mucosa of the nose and mouth as well as bone fractures, can be produced in mice within a short period using vitamin A, vitamin A acid or its derivatives. The hypervitaminosis A test was carried out on mice weighing 25–27 g which over a 14-days' period received 10 i.p. injections of the test substance suspended in arachis oil. The following symptoms were evaluated using a scale from 0 to 4. Loss of weight: > 10 g = 4, 7–9 g = 3, 4–6 g = 2, 1–3 g = 1, < 1 g = 0. Skin scaling: very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0. Loss of hair: very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0. Bone fractures of the extremities (number, macroscopic observation): 4 or > 4 = 4, 3 = 3, 2 = 2, 1 = 1, none = 0. The lowest daily dose able to cause a hypervitaminosis A in the

14-days' study was specified. Hypervitaminosis A was defined as being that condition of the animals where the addition of all the symptom grades yielded at least 3.

Therapeutic ratio of the antipapilloma activity

The limiting factor in the therapy with vitamin A preparations is the so-called hypervitaminosis A. In order to define preparations as concerns antipapilloma activity and toxicity, a therapeutic ratio was calculated which was determined from the weekly i.p. dose causing in the 14 days' therapeutic test approximately 50 % papilloma regression, and from the lowest daily i.p. dose producing after 14 days hypervitaminosis A symptoms, as defined above. This provides a picture of the therapeutic margin of a retinoic acid analog. A dissociation between antipapilloma activity and hypervitaminosis A was sought. Calculation of the "Therapeutic Ratio" (T.R.):

$$\text{T.R.} = \frac{\text{Papilloma regression: ED 50 \%/week}}{\text{Hypervitaminosis A: min. dose/day}}$$

Transplantable tumors

I was tested for its effect on 4 transplantable tumors: Ehrlich carcinoma (solid form), Ehrlich ascites carcinoma, Crocker sarcoma S 180 and leukemia L1210. Five animals were used for the controls and for each dosage group. In each case, treatment was started on the day of tumor implantation. I was injected i.p. as a fine suspension in arachis oil in doses of 100–400 mg/kg once a week. Changes in the weight of the mice were recorded.

Ehrlich carcinoma, solid form, in Swiss albino mice. Subcutaneous implantation of 10^7 Ehrlich ascites tumor cells. Excision of the tumors on the 8th day.

Ehrlich ascites carcinoma, in Swiss albino mice. I.p. implantation of 10^7 Ehrlich ascites tumor cells.

Crocker sarcoma S 180, solid form, in Swiss albino mice. S.c. implantation of 0·5 ml of a 50 % tumorbrei suspension. Excision of the tumors on the 10th day.

Leukemia L1210, in BDF_1-hybrids (DBA ×

Table 1. *Results of treatment of skin papillomas for* 2 *weeks with retinoic acid and its analog I*

Dose (mg/kg)	Average sum of the papilloma dia/animal (mm)		Per cent change in the average sum of the papilloma dia/animal
	Day 0	Day 14	
Controls	21·1	26·0	+23·2%
Retinoic acid			
400 1 × weekly i.p.	21·6	11·2	−48·2%
200	24·7	15·8	−36·0%
Analog I			
200 1 × weekly i.p.	25·5	6·6	−74·1%
100	28·0	8·5	−69·6%
50	20·4	9·1	−55·4%
25	24·4	12·5	−48·8%
12·5	24·8	17·3	−30·2%
Analog I			
400 1 × weekly p.o.	22·0	4·8	−78·2%
200	23·7	9·8	−58·7%
100	21·5	14·3	−33·5%
50	21·0	16·5	−21·4%
25	16·0	14·2	−11·3%
Analog I			
40 daily p.o.	19·5	4·6	−76·4%
20	16·6	7·4	−55·4%
10	18·4	9·8	−46·7%
5	16·5	13·7	−17·0%

Comparing the average sums of diameters on day 0 and day 14, the *t*-test showed significant decreases for the treated animals ($P < 0{\cdot}05$). Furthermore, there exists an increasing reduction of the papilloma diameters as a function of the dose with significant regression lines.

C57BL). Intraperitoneal implantation of 10^5 cells from leukemic ascites.

In the animals with Ehrlich solid carcinoma and Crocker sarcoma S 180, the tumor-weight index was determined, whereas in those with Ehrlich ascites carcinoma and leukemia L1210, the survival-time index served as a parameter for the efficacy of the treatment. Evaluation was based on mean values of each group.

RESULTS

1. *Chemically induced skin tumors*

Skin papillomas. As can be seen from Table 1, skin papillomas can be made to regress clearly with I in a dose-dependent way. After i.p. administration of 200 mg/kg I once a week, there is a reduction of 74·1 % of the papilloma diameters within 14 days. In order to achieve a similar result with oral administration, it was necessary to double the dose to 400 mg/kg. Intraperitoneal administration of doses of 50 mg/kg once a week still lead to a decrease in the papilloma diameters by 55·4 %. Oral doses, either 200 mg/kg once a week or 20 mg/kg daily are necessary for obtaining approximately the same regression of tumors. When an equal total amount of I was given, the i.p. injections were superior to the oral administrations and within the frame of the oral mode of administration, the daily applications were superior to the weekly ones.

Skin carcinomas. The results can be seen in Table 2. All 11 control animals displayed tumor growth within the two weeks' test phase. The growth rate was very variable and fluctuated between a minimum progression of 14·3 % and a maximum of 316·5 % with an average progression of 107·4 %. The treated animals reacted very differently to the administration of I. Two of the 11 treated mice showed carcinoma growth despite the therapy. The remaining 9 animals responded more or less well to the therapy. Whereas the least affected tumor regressed by only 13·9 % of its volume, the carcinoma volume in 3 animals was reduced by 90·9, 96·4 and 97·2 %. The volume of the carcinomas of the treated animals showed an average regression of 36·0 %.

2. *Hypervitaminosis A*

Retinoic acid produces a hypervitaminosis A in mice after daily i.p. administration of doses of 80 mg/kg. I evokes the same syndrome in daily doses of 50 mg/kg i.p.

3. *Therapeutic ratio*

The therapeutic ratio is represented by the quotient between the weekly i.p. dose leading

Table 2. Results of treatment of skin carcinomas for 2 weeks with the retinoic acid analog I

	Carcinoma volume per animal (mm^3)		Per cent change in volume
	Day 0	Day 14	
Controls	605	2020	+316·5
	260	780	+200·0
	576	1710	+196·9
	180	440	+144·4
	540	1050	+ 94·4
	420	784	+ 86·7
	96	144	+ 50·0
	490	640	+ 30·6
	896	1120	+ 25·0
	1938	2376	+ 22·6
	1260	1440	+ 14·3
Analog I			
400 mg/kg 1 × weekly p.o.	120	286	+138·3
	528	720	+ 36·4
	975	840	− 13·9
	324	216	− 33·3
	1092	676	− 38·1
	240	126	− 47·5
	588	200	− 66·0
	384	48	− 87·5
	330	30	− 90·7
	350	12·5	− 96·4
	572	16	− 97·2

Table 3. Comparison of the therapeutic ratio of retinoic acid and its analog I

Compound	Hypervitaminosis A min. dose/day	Papilloma regression ED 50%/week	Therapeutic ratio
Retinoic acid	80 mg/kg	400 mg/kg	$\frac{400}{80} = 5$
Analog I	50 mg/kg	25 mg/kg	$\frac{25}{50} = 0{\cdot}5$

The lower the quotient, the more favorable the therapeutic ratio.

Table 4. Results of treatment of transplantable tumors with the retinoic acid analog I

4.1. Ehrlich carcinoma, solid form

Dose of I mg/kg i.p. 1 × weekly	Tumor wt (mg)	Mouse wt (change in g)	Tumor wt index C/T (Controls: treated animals)
Controls	343	+2·2	1·0
100	351	−0·2	0·98
200	327	−0·6	1·05
400	340	−1·4	1·01

4.2. Ehrlich ascites carcinoma

Dose of I mg/kg i.p. 1 × weekly	Survival time (days)	Survival time index T/C (Treated animals: controls)
Controls	12·0	1·0
100	15·8	1·15
200	12·4	1·03
400	12·6	1·05

4.3. Crocker sarcoma S 180

Dose of I mg/kg i.p. 1 × weekly	Tumor wt (mg)	Mouse wt (change in g)	Tumor wt index C/T (Controls: treated animals)
Controls	.1530	+1·0	1·0
100	1539	+0·6	0·99
200	1406	+0·4	1·09
400	1527	+0·6	1·0

4.4. Leukemia L 1210

Dose of I mg/kg i.p. 1 × weekly	Survival time (days)	Survival time index T/C (Treated animals: controls)
Controls	8·6	1·0
100	8·4	0·98
200	8·0	0·93
400	7·6	0·88

The calculated regression lines showed no significant dependence of the response variables (tumor weight index and survival time index) on the dose (4.1. $P > 0{\cdot}5$, 4.2. $P > 0{\cdot}5$, 4.3. $P > 0{\cdot}5$, 4.4. $P > 0{\cdot}1$). A comparison of the response values of the treated animals with the control value 1·0 by means of the *t*-test showed also no significant difference (4.1. $P > 0{\cdot}5$, 4.2. $P > 0{\cdot}17$, 4.3. $P > 0{\cdot}45$, 4.4. $P > 0{\cdot}13$).

to an approximate 50 % regression of papillomas and the lowest daily i.p. dose causing hypervitaminosis A symptoms. The lower the ratio, the better the preparation, i.e. the more active and the less toxic it is. The results of the comparison of the therapeutic ratios of retinoic acid and its aromatic analog I are presented in Table 3. From this table it can be seen that I possesses a therapeutic ratio ten times more favorable than retinoic acid.

4. *Transplantable tumors*

I exerted no influence whatsoever on the growth of the 4 examined transplantable tumors (Table 4).

DISCUSSION

The aromatic retinoic acid analog I has the same properties, qualitatively as retinoic acid. It causes a regression of chemically induced skin papillomas and carcinomas. The growth of transplantable tumors, however, is not inhibited. This compound, like retinoic acid, does not act in the same way as the well-known cytotoxic agents. It does not interfere generally with the proliferation of growing tissues by depressing the mitotic cycle. Thus, it does not influence the rapidly growing undifferentiated transplantable tumors nor does it inhibit other tissues with a high proliferation rate such as the hemopoietic tissues. It has a more specific activity probably in connection with its affinity to epithelial tissues.

Retinoic acid, as well as its aromatic analog I, produces the hypervitaminosis A syndrome. From the quantitative point of view, they behave quite differently. This is expressed by their varying ratios between papilloma regression and the side effects—the so-called hypervitaminosis A. The aromatic analog I possesses a therapeutic relationship which is ten times more favorable than that of retinoic acid. This superiority was established with the model of the skin papilloma of mice, a precancerous condition, as this benign epithelial tumor has a distinct tendency to malignant transformation into carcinoma. It cannot be predicted whether this advantage of the analog I is also true for precancerous conditions of mucous membranes of the gastrointestinal, respiratory and genitourinary tract. Furthermore, other species may react differently. However, it seems justified that clinical investigations are undertaken to obtain information as to whether precancerous conditions in man will respond to this retinoic acid analog.

The following lesions may fall into this category of preneoplasias: actinic keratoses of the skin, leukoplakias of mouth, tongue, larynx and vulva, dysplasias of the cervix, papillomas of the urinary bladder and premalignant changes of the bronchial epithelium of uranium and asbestos workers or heavy smokers.

Concerning the mechanism of action, I has an effect on epithelium differentiation similar to that of retinoic acid. In the modified colpokeratosis test of Boguth *et al.* [7], retinoic acid and its analog I had a similar preventive effect on the squamous metaplasia of the vaginal epithelium produced by hormones [8]. Sporn [9] was able to demonstrate a squamous metaplasia preventing effect on the tracheobronchial epithelium in an organ culture model system with retinol, retinoic acid and its analog I. We do not know yet whether the prevention or even reversion of the hyperplasias, dysplasias and squamous metaplasias induced by vitamin A deficiency, hormones or carcinogenic hydrocarbons [10–14] is based on the same phenomenon as the regression of chemically induced established benign or malignant epithelial tumors.

This investigation has shown that using the *in vivo* models of skin papillomas and hypervitaminosis A, it is possible to screen and evaluate quantitatively retinoic acid analogs. The therapeutic ratio enables us to compare various analogs and to select the best one for clinical trials. The distinct difference between the therapeutic ratio of retinoic acid and that of the aromatic analog I allows the conclusion that the anti-papilloma effect and the hypervitaminosis A producing effect are not strictly linked together. Therefore, it lies in the realm of possibility that compounds exhibiting an even more marked dissociation between the advantageous antitumor activity and the dose limiting toxic side effects of hypervitaminosis A may be still better tools for the experimental and clinical prophylaxis and therapy of premalignant and malignant epithelial conditions.

REFERENCES

1. W. Bollag, Effects of vitamin A acid on transplantable and chemically induced tumors. *Cancer Chemother. Rep.* **55,** 53 (1971).

2. W. Bollag, Vitamin A-Säure in der Tumortherapie. Tierexperimentelle Untersuchungen über die Wirkung von Vitamin A-Säure auf Tumoren. *Schweiz. med. Wschr.* **101,** 11 (1971).
3. W. Bollag, Prophylaxis of chemically induced benign and malignant epithelial tumors by vitamin A acid (retinoic acid). *Europ. J. Cancer* **8,** 689 (1972).
4. W. Bollag and F. Ott, Therapy of actinic keratoses and basal cell carcinomas with local applications of vitamin A acid. *Cancer Chemother. Rep.* **55,** 59 (1971).
5. H. J. Ryssel, K. W. Brunner and W. Bollag, Die perorale Anwendung von Vitamin A-Säure bei Leukoplakien, Hyperkeratosen und Plattenepithelkarzinomen: Ergebnisse und Verträglichkeit. *Schweiz. med. Wschr.* **101,** 1027 (1971).
6. J. P. Evard and W. Bollag, Konservative Behandlung der recidivierenden Harnblasenpapillomatose mit Vitamin A-Säure. Vorläufige Mitteilung. *Schweiz. med. Wschr.* **102,** 1880 (1972).
7. W. Boguth, V. Horn, M. K. Solliman and H. Weiser, Eine neue Methode zur Bestimmung kurz wirksamer Vitamin A-Präparate. *Int. J. Vitamin Res.* **31,** 6 (1960).
8. H. Weiser, (Personal communication).
9. M. B. Sporn, (Personal communication).
10. G. H. Clamon, M. B. Sporn, J. M. Smith and U. Saffiotti, α- and β-retinyl acetate reverse metaplasias of vitamin A deficiency in hamster trachea in organ culture. *Nature* **250,** 64 (1974).
11. I. Lasnitzki, Growth pattern of the mouse prostate gland in organ culture and its response to sex hormones, vitamin A and 3-methylcholanthrene. *Nat. Cancer Inst. Monogr.* **12,** 381 (1963).
12. I. Lasnitzki and De Witt S. Goodman, Inhibition of the effects of methylcholanthrene on mouse prostrate in organ culture by vitamin A and its analog. *Cancer Res.* **34,** 1564 (1974).
13. U. Saffiotti, R. Montesano, A. R. Sellakumar and S. A. Borg, Experimental cancer of the lung. Inhibition by vitamin A of the induction of tracheobronchial squamous metaplasia and squamous cell tumors. *Cancer* **20,** 857 (1967).
14. W. Bollag, Vitamin A and vitamin A acid in the prophylaxis and therapy of epithelial tumors. *Int. J. Vitamin Res.* **40,** 299 (1970).

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Suppression of Mitogen Responses by Serum from Tumour-bearing Mice*

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Abstract—*Stimulation of DBA/2J mouse spleen cells by the T-cell mitogen, concanavalin A (conA), and the B-cell mitogen, lipopolysaccharide (LPS), was obtained in medium supplemented with isologous serum. The system was used to investigate the effects of serum from tumour-bearing animals on these mitogen responses. It was found that serum from mice with large tumours was markedly inhibitory to the response of normal spleen cells to both LPS and conA. The degree of inhibitory activity of the serum was directly related to the tumour-load of the serum donor. The results are considered in relation to the well known generalized immunosuppression which occurs in individuals with large tumours.*

INTRODUCTION

MOUSE lymphoid cells have been routinely cultured and stimulated by mitogens, antigens and allogeneic cells in the presence of heterologous sera such as human or fetal calf [1–4]. Also recent reports have described systems for stimulation in serum-free medium [5–7]. However, successful stimulations in the presence of mouse serum have not been reported except for a recent publication in which mouse mixed lymphocyte reactions were achieved in the presence of isologous serum [8].

Since mice are the subjects of many basic tumour studies, the ability to culture mouse lymphoid cells in the presence of isologous serum could be useful in investigating the mechanism of the immunosuppression that is commonly induced by progressive tumour growth [9–13]. The existence of specific serum blocking factors which can inhibit tumour-specific responses *in vitro* and which might be responsible for the depression of tumour-specific immunity often associated with progressive tumour growth [10–13] has been well documented. The general depression of other responses which are not tumour-specific [9, 10] may be related to other serum components which could be detected in a lymphocyte stimulation system. Previous work in human systems has been inconclusive regarding the effect of serum from tumour-bearing individuals on mitogen stimulation [16–18], a phenomenon which may be used as an indicator of general cellular competence.

The present studies have shown that mitogen stimulation of mouse spleen cells can be achieved in the presence of isologous serum. The development of this system has made it possible to assess the effects of serum from mice bearing tumours of various sizes on the stimulation of normal spleen cells by conA, a T-cell mitogen, and LPS, a B-cell mitogen [3, 19, 20].

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MATERIAL AND METHODS

Mice

DBA/2J female mice (Jackson Laboratories, Bar Harbor, Maine) aged 2–4 months were used exclusively in these studies.

Tumour system

A methylcholanthrene-induced rhabdomyosarcoma of DBA/2J mice was obtained from

Jackson Laboratories and was passaged both *in vitro* and *in vivo* in our laboratory. Tumours were grown subcutaneously on the animal's right abdominal side. Inoculation of 10^4 tumour cells produced tumours in 100% of recipients. Previous studies have shown that this tumour is strongly immunogenic. Mice immunized by tumour induction and subsequent surgical excision routinely rejected a secondary challenge of 10^5 tumour cells. The tumour cells were also maintained *in vitro* on 100 × 20 mm plastic Falcon petri dishes (#3003) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) both of which were obtained from Grand Island Biological Company (GIBCO), Grand Island, New York. The medium also contained penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (50 μg/ml). The cultures were kept at 37°C in a humidified atmosphere of 95% air—5% CO_2. They were passaged every 4–7 days by trypsinization (0·25%, GIBCO, 5 min incubation followed by wash with serum) and transfer to new plates. Full details of this tumour system have been described elsewhere [10].

Collection and preparation of serum

Blood was collected from the retro-orbital sinus of normal mice and mice bearing tumours of various sizes. The blood was allowed to clot overnight at 4°C after which the serum was separated and centrifuged. Sera were inactivated at 56°C for 30 min and stored at −20°C.

Serum was collected at the following stages of tumour growth: Group A—tumours not yet palpable (6 days after inoculation of 10^4 cells); Group B—tumours of about 0·60–1·0 g (about 3 weeks after inoculation); Group C—tumours about 1·8–2·2 g (about 6 weeks after inoculation); Group D—tumours > 2·5 g about 7–8 weeks after inoculation.

Culture system and mitogen stimulation

RPMI-1640 medium alone or supplemented with different concentrations of mouse serum and/or FCS was used exclusively. It was found previously that Eagle's minimal essential medium (MEM) would not support mitogen stimulation without serum, or in the presence of mouse serum. The method of culture was basically the micromethod described by Hartzman *et al.* [21]. In brief, pooled spleen cells from 3 or more normal mice (5×10^5/0·2 ml medium) were dispensed to wells of microtitre plates (Linbro Chemical Co., New Haven, Connecticut). Mitogens were added in 0·05 ml volumes to appropriate wells to the following final concentrations: concanavalin A (conA, 1·0 μg/ml) and lipopolysaccharide W (LPS, 25 μg/ml). ConA was obtained from Sigma Chemical Co., St. Louis, Missouri, and LPS (*E. coli* endotoxin) came from Difco Laboratories, Detroit Michigan. Cultures were maintained from 1–5 days as indicated for each experiment (normally 2 days) at which time ^{3}H-thymidine (2 Ci/mmole, Amersham-Searle, Don Mills, Ontario) was added in a 0·05 ml volume (1·0 μCi/well). Sixteen hours after isotope addition the cultures were harvested by a micro-precipitator apparatus which aspirated the contents of one row of 12 wells and deposited the contents in discrete spots on a glass fiber strip. Each spot was cut out, placed in a glass scintillation vial with 3.0 ml of toluene-based (4.0 g omnifluor/l. toluene) scintillation fluid and counted with a Nuclear Chicago Unilux 1 scintillation counter.

Statistics

Results are expressed as mean values ± the S.E. of the mean. The statistical significance of differences in mean values was determined by Student's *t*-test. Differences were considered to be significant if the probability that the observed difference occurred by chance alone was less than 5% (i.e. $P < 0{\cdot}05$).

RESULTS

Both conA and LPS caused a significant stimulation of ^{3}H-thymidine incorporation by normal spleen cells in serum-free medium and in medium supplemented with various concentrations of normal isologous serum (Fig. 1). ConA stimulation was maximum with 1% serum. The response decreased at higher serum concentrations but was still well above unstimulated levels even at 4% serum. The LPS response was maximum at 0·12% serum and generally decreased slightly as the serum concentration was increased No LPS stimulation was obtained with 4% serum.

Figure 2 shows the time-course of stimulation by conA (A) and LPS (B) in serum-free medium, medium supplemented with 5% FCS, and medium supplemented with 1% normal isologous serum. Stimulation by conA in the presence of isologous serum was maximum at days 2 and 3. In serum-free medium the peak response was reached at days 3 and 4. During the first 3 days the level of stimulation was greater with isologous serum, but on days 4 and 5 stimulation was equal or greater with serum-free medium. The conA response with FCS was maximum at day 2 and rapidly declined thereafter to below serum-free levels

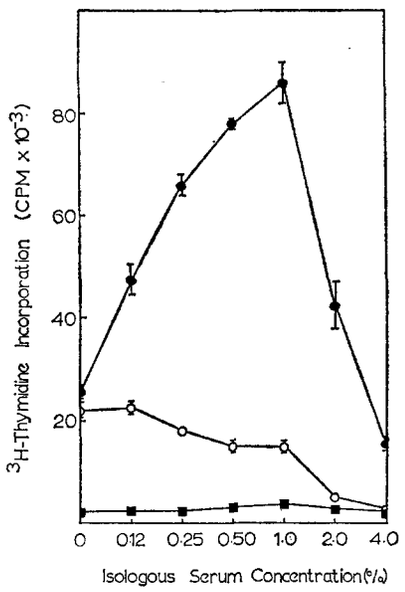


Fig. 1. *Effect of normal isologous mouse serum concentration on mitogen stimulation.* ●——●, *conA;* ○——○, *LPS;* ■——■, *unstimulated. Spleen cells were cultured in serum-free medium or in medium supplemented with various concentrations of normal isologous serum for* 2 *days.* ^{3}*H-thymidine was then added, and the cultures were harvested* 16 *hr later. Each point is the mean counts/min* ± *S.E.M. of* 3–4 *replicate cultures.*

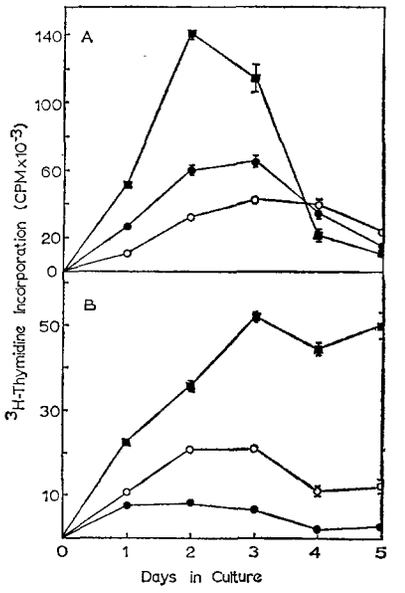


Fig. 2. *Time course of mitogen stimulation.* ●——●, 1% *normal isologous mouse serum;* ○——○, *serum-free;* ■——■, 5% *FCS. ConA was the mitogen in* (*A*) *and LPS in* (*B*). ^{3}*H-thymidine was added to a set of cultures at each of the times indicated on the figure* (1, 2, 3, 4 *or* 5 *days after mitogen addition*), *and the cultures were harvested* 16 *hr later. Each point is the mean counts/min* ± *S.E.M. of* 3–4 *replicate cultures. Mean unstimulated values for each protocol for days* 1–5 *respectively were as follows: serum-free:* 3011, 3089, 1878, 1008, 2058; *isologous serum:* 1999, 1879, 1272, 1680, 2942; 5% *FCS:* 3150, 5409, 5685, 5791, 6581.

by day 4. The maximum absolute level of stimulation with FCS was about 2 times that obtained with mouse serum, but the unstimulated levels were also about 2 times as great. LPS responses were consistently higher in serum free medium than in medium containing 1% isologous serum. The responses in serum-free medium and medium with isologous serum might have been more comparable if 0·12% serum, which produced the maximum response (Fig. 1), had been used. However, since the difference between the responses with 0·12 and 1·0% serum was so small and since tumour sera effects could be studied at 1·0 but not 0·12%, the former concentration was judged to be more significant to the present purpose. The LPS response was reduced to stimulated levels on days 4 and 5 in the presence of isologous serum. Stimulation in FCS medium reached a maximum at day 3 and remained high through day 5.

Sera were obtained and pooled from groups of mice at 4 stages of tumour development and were tested for their effects on mitogen stimulation of normal spleen cells in comparison to normal serum. Serum from group A (tumours not yet palpable) significantly enhanced the conA response above the level observed with normal serum at all concentrations above 0·50% (Fig. 3A). However, it should be noted

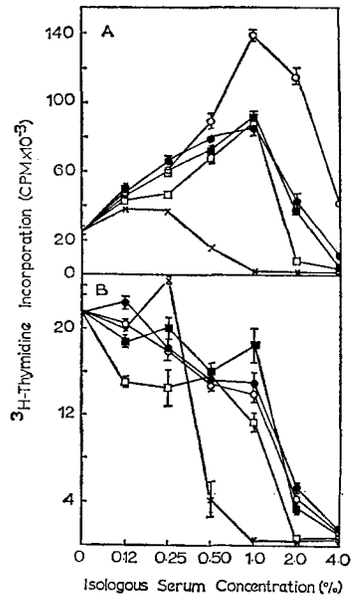


Fig. 3. *Effect of concentration of isologous serum from mice bearing tumours of various sizes* (*see Methods*) *on mitogen stimulation.* ●——●, *normal serum;* ○——○, *group A serum;* ■——■, *group B serum;* □——□, *group C serum;* ×——×, *group D serum. ConA was the mitogen in* (*A*) *and LPS in* (*B*). ^{3}*H-thymidine was added at* 2 *days, and cultures were harvested* 16 *hr later. Each point is the mean counts/min* ± *S.E.M. of* 3–4 *replicate cultures. The S.E.M. was less than the size of the points for each entry of group D.*

that in some experiments Group A serum did not have such a strong enhancing effect. Group B (0·60–1·0 g tumours) and group C (1·9–2·2 tumours) sera were equivalent to normal serum up to concentrations of 4·0 and 2·0% respectively at which points they significantly reduced the response below the normal level. Group D serum (tumours > 2·5 g) was significantly inhibitory at 0·12%, and it progressively decreased the response at higher concentrations. Inhibition to unstimulated levels occurred with 1% serum.

The effects of these sera on the LPS response were somewhat different (Fig. 3B). Serum of mice from group A was identical to normal sera at all concentrations. No enhancing effect was observed as was seen for the conA response. Group D serum was significantly inhibitory to LPS stimulation only at 0·50% and higher. Sera from B and C mice were inhibitory only at concentrations above 2·0 and 1·0% respectively.

The inhibition of the mitogen responses by the tumour sera relative to normal sera did not appear to be due predominantly to cytotoxic effects as measured by dye exclusion by unstimulated cells (Table 1). The most effective inhibitory sera (group D) was no more toxic than normal sera in the absence of FCS and was less toxic than normal sera in the presence of FCS. A much greater percentage of cells died in serum-free medium than in medium with 4% isologous serum. It should be realized though, that the dye exclusion tests are not absolutely conclusive since the cells could be functionally killed. Further studies in progress will clarify this point.

The presence of the various tumour sera at 1% concentration did not alter the time-course of conA (Fig. 4A) or LPS (Fig. 4B) stimulation. Peak responses were generally observed on day 2 except with D serum which completely inhibited both conA and LPS stimulation at all times. Group A serum marginally enhanced the conA response above the level obtained with normal serum on the first 3 days. Both B and C sera reduced the conA response by from 30 to 50% at all times. They had a lesser effect on the LPS response.

Table 1. Viabilities of unstimulated spleen cells cultured with various sera

Serum* composition of medium	% Viable† Time in culture (days) 2	3	4
OFCS + none	37	25	11
OFCS + 4% normal	46	61	45
OFCS + 4% group D	39	52	44
5% FCS + none	49	65	73
5% FCS + 4% normal	49	34	40
5% FCS + 4% group D	57	51	64

*Normal spleen cells were cultured in RPMI-1640 medium alone or supplemented with 5% FCS and/or 4% isologous serum from either normal or tumour-bearing (group D, > 2·5g) mice.

†The percentage of viable cells was determined at the indicated times by a dye exclusion test using trypan blue. The cells were cultured in tubes at $2·5 \times 10^6$ cells/ml and aliquots were removed at the desired times. An equal volume of dye was added, and the cells were counted in a hemocytometer.

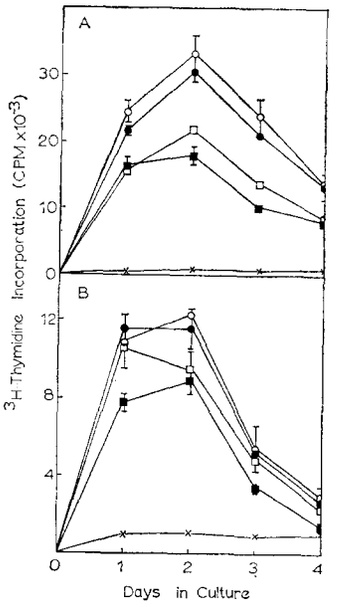


Fig. 4. Time course of mitogen stimulation in presence of isologous serum from mice with tumours of various sizes. ●——●, *normal serum;* ○——○, *group A serum;* ■——■, *group B serum;* □——□, *group C serum;* ×——×, *group D serum. ConA was the mitogen for (A) and LPS for (B). The serum concentration was 1% in all cases.* ^{3}*H-thymidine was added at the indicated times, and harvesting was done 16 hr later. Each point is the mean counts/min ± S.E.M. of 3–4 replicate cultures.*

The effects of tumour-bearer serum on stimulations were also tested using medium containing 5% FCS. The ^{3}H-thymidine incorporation in the presence of each type of tumour-bearer's serum at a given concentration is normalized by expressing it as a percentage of the incorporation obtained with the same concentration of normal serum. Significant inhibition of conA stimulation was observed at 4·0, 2·0, 1·0 and 0·5% for groups A, B, C and D respectively (Fig. 5A). Inhibition of the LPS response was not significant for A, B, or C serum except at 2·0 and 4·0% (Fig. 5B).

Serum from Group D caused inhibition at 1·0 % and completely blocked stimulation at 2 %.

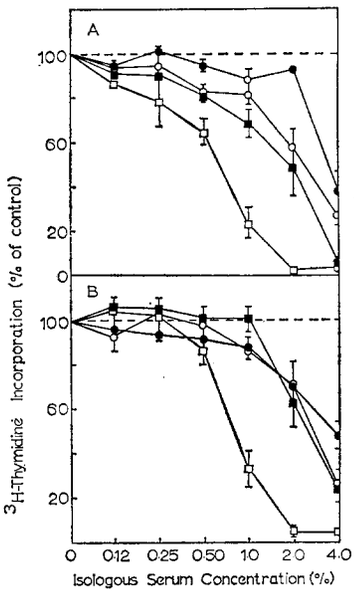


Fig. 5. Effect of various concentration of isologous serum from mice with tumours of different sizes on mitogen stimulation in medium supplemented with 5 fetal calf serum. ●——●, group A serum; ○——○, group B serum; ■——■, group C serum; □——□, group D serum. ConA was the mitogen in (A) and LPS in (B). ^{3}H-thymidine was added at 2 days, and cultures were harvested 16 hr later. Results are expressed as a percentage of isotope incorporated in presence of various tumour serums compared to that incorporated in presence of an equivalent concentration of control normal isologous serum. Each point represents the mean % ± S.E.M. of 4 separate experiments. Each experiment consisted of 3–4 replicate cultures for each point.

DISCUSSION

The present results demonstrated that mouse spleen cells can be stimulated by mitogens in medium supplemented with low concentrations of isologous serum. This observation made it possible to assess the effects of serum from tumour-bearing mice on mitogen stimulation. It was found that the serum from mice bearing a methylcholanthrene-induced rhabdomyosarcoma was inhibitory to mitogen stimulation of normal spleen cells, and the degree of inhibition was directly related to tumour load. Previous clinical studies have shown that plasma from patients with a variety of solid tumours inhibited stimulation of normal peripheral blood lymphocytes by the mitogen, phytohemagglutinin (PHA) [16, 22], although there have been contradictory reports [17, 18]. A study appearing during the course of the present investigations demonstrated that serum from patients with nonlymphoid tumours inhibited stimulations of blood lymphocytes by both PHA and allogeneic cells and, like our results, the degree of inhibition was related to the stage of the disease [23].

Since mitogen stimulation provides a measure of the general functional capacity of lymphocytes, the implication of this evidence is that individuals with solid tumours may have a factor in their serum which inhibits normal lymphocyte function. The existence of such a substance could explain the frequent observations of depressed cell-mediated immunity in tumour-bearing patients [22] and mice [9, 10]. Also, our previous studies showed that mitogen responses, both conA and LPS, decreased with increasing tumour load correlating exactly with the increased inhibitory effectiveness of the tumour-bearers' serum [10]. Several other animal studies have indicated the presence of an immunosuppressive substance(s) in tumour bearers. Chan and Sinclair observed that serum and cell-free ascites fluid from mice with radiation-induced lymphomas grown in ascites form inhibited development of antibody-forming cells *in vitro* [25]. Two other groups have found that treatment of normal mice with cell-free ascites fluid from Ehrlich carcinoma inhibited formation of antibody-producing cells against sheep erythrocytes [26, 27].

Of particular interest in the present studies was the observation that tumour serum inhibited stimulation by both conA and LPS. Since it has been shown relatively convincingly that conA and LPS are T-and B-cell stimulators respectively in mice [3, 19, 20], it appears that the inhibitory substance affects both lymphocyte subpopulations. This was expected from our previous results showing that mice with large tumours had depressed responses to both mitogens [10].

Another observation made here which cannot be explained at present was the enhancement of the conA but not the LPS response above the level obtained with normal serum when the cells were exposed to serum from mice which had been injected with tumour cells but which had not yet developed palpable tumours. However, of possible relevance to this finding was the recent report that autologous serum from adult patients with acute leukemia in some cases enhanced stimulations by autologous leukemia cells and PHA, while in other cases it was inhibitory [28].

It is well-established that many tumour hosts have serum factors which can specifically block anti-tumour responses *in vitro* [29–31]. The present work and other studies mentioned above certainly indicate the presence in tumour-

bearer serum of a factor(s) which generally inhibits lymphocyte function. What relation, if any, exists between them will require considerable further study. Although more attention has been given to studying the tumour-specific inhibitory factors, it could well be that the non-specific ones are of equal or greater importance since the main cause of death in cancer patients is not the tumour itself but the accompanying generalized infections [32] which likely result from the generalized immunosuppression.

REFERENCES

1. W. H. Adler, T. Takiguchi, B. Marsh and R. T. Smith, Cellular recognition by mouse lymphocytes *in vitro*—I. Definition of a new technique and results of stimulation by phytohemagglutinin and specific antigens. *J. exp. Med.* **131,** 1049 (1970).
1. S. M. Phillips, C. B. Carpenter and J. P. Merrill, Cellular immunity in the mouse I. *In vitro* lymphocyte reactivity. *Cell Immunol.* **5,** 235 (1973).
3. J. Andersson, G. Möller and O. Sjöberg, Selective induction of DNA synthesis in T and B lymphocytes. *Cell Immunol.* **4,** 381 (1972).
4. K. Shortman, W. J. Byrd, J. C. Cerottini and K. T. Brunner, Characterization and separation of mouse lymphocyte subpopulations responding to phytohemagglutinin and pokeweed mitogens. *Cell. Immunol.* **6,** 25 (1973).
5. E. Gazit and T. N. Harris, Mixed leucocyte culture with mouse spleen cells in a serum-free medium. *Proc. Soc. exp. Biol.* **140,** 750 (1972).
6. E. Heber-Katz, A. B. Peck and R. E. Click, Immune responses *in vitro*. Mixed leukocyte interactions in a protein-free medium. *Europ. J. Immunol.* **3,** 379 (1973).
7. A. Coutinho, G. Möller, J. Andersson and W. W. Bullock, *In vitro* activation of mouse lymphocytes in serum-free medium: effect of T and B cell mitogens on proliferation and antibody synthesis. *Europ. J. Immunol.* **3,** 299 (1973).
8. A. B. Peck and R. E. Click, Immune responses *in vitro*—III. Enhancement of the mouse mixed lymphocyte interaction by isologous and homologous sera. *Europ. J. Immunol.* **3,** 385 (1973).
9. W. H. Adler, T. Takiguchi and R. T. Smith, Phytohemagglutinin unresponsiveness in mouse spleen cells induced by methylcholanthrene sarcomas. *Cancer Res.* **31,** 864 (1971).
10. R. B. Whitney, J. G. Levy and A. G. Smith, The influence of tumour size and surgical resection on cell-mediated immunity in mice. *J. nat. Cancer Inst.* (In press).
11. D. LeFrancois, J. K. Youn, J. Belehradek and G. Barski, Evolution of cell-mediated immunity in mice bearing tumours produced by a mammary carcinoma cell line. Influence of tumour growth, surgical removal, and treatment with irradiated tumour cells. *J. nat. Cancer Inst.* **46,** 981 (1971).
12. P. J. Deckers, R. C. Davis, G. A. Parker and J. A. Mannick, The effect of tumour size on concomitant tumour immunity. *Cancer Res.* **33,** 33 (1973).
13. R. C. Rees and C. W. Potter, Immune response to adenovirus 12-induced tumour antigens, as measured *in vitro* by the macrophage migration inhibition test. *Europ. J. Cancer* **9,** 497 (1973).
14. I. Hellstrom and K. Hellstrom, Colony inhibition and cytotoxicity assays. In *In Vitro Methods in Cell-Mediated Immunity*. (Edited by B. R. Bloom and P. R. Glade), p. 409. Academic Press, New York (1971).
15. R. W. Baldwin and M. J. Embleton, Demonstration by colony inhibition methods of cellular and humoral immune reactions to tumour-specific antigens associated with aminoazo-dye-induced rat hepatomas. *Int. J. Cancer* **7,** 17 (1971).
16. M. Silk, Effect of plasma from patients with carcinoma on *in vitro* lymphocyte transformation. *Cancer* **20,** 2088 (1967).
17. E. K. Golub, T. Israsena, A. C. Quatrale and K. L. Becker, Effect of serum from cancer patients on homologous lymphocyte cultures. *Cancer* **23,** 306 (1969).
18. M. Al-Sarraf, S. Sardesai and V. K. Vaitkevicius, Effect of syngeneic and allogeneic plasma on lymphocytes from cancer patients, patients with non-neoplastic diseases, and normal subjects. *Cancer* **27,** 1426 (1971).

19. J. ANDERSSON, O. SJÖBERG and G. MÖLLER, Induction of immunoglobulin antibody synthesis *in vitro* by lipopolysaccharides. *Europ. J. Immunol.* **2,** 349 (1972).
20. I. GERY, J. KRUGER and S. Z. SPIESEL, Stimulation of B-lymphocytes by endotoxin. Reactions of thymus-deprived mice and karyotypic analysis of dividing cells in mice bearing T6 T6 thymus grafts. *J. Immunol.* **108,** 1088 (1972).
21. R. J. HARTZMAN, M. SEGAL, M. L. BACH and F. H. BACH, Histocompatibility matching VI. Miniaturization of the mixed leukocyte culture test: a preliminary report. *Transplant. Rev.* **11,** 268 (1971).
22. R. C. BAGAI, T. H. M. STEWART and J. E. HARRIS, Use of plasma-free medium for the *in vitro* culture of human lymphocytes. *Cancer* **31,** 882 (1973).
23. N. SUCUI-FOCA, J. BUDA, J. MCMANUS, J. THIEM and K. REEMTSMA, Impaired responsiveness of lymphocytes and serum inhibitory factors in patients with cancer. *Cancer Res.* **33,** 3473 (1973).
24. E. M. HERSH, J. U. GUTTERMAN and G. MAVLIGIT, *Immunotherapy of Cancer in Man*, pp. 3–15, Academic Press, New York (1973).
25. P. L. CHAN and N. R. ST. C. SINCLAIR, Immunosuppressive factors associated with tumours. In *Microenvironmental Aspects of Immunity.* (Edited by B. D. JANHOVIC and K. ISAHOVIC), p. 469. Plenum, New York (1973).
26. H. YAMAZAKI, K. NITTA and H. UMEZAWA, Immunosuppression induced with cell-free fluid of Ehrlich carcinoma ascites and its fractions. *Gann* **64,** 83 (1973).
27. I. HRSAK and T. MAROTTI, Immunosuppression mediated by Ehrlich ascited fluid. *Europ. J. Cancer* **10,** 717 (1973).
28. J. U. GUTTERMAN, E. M. HERSH, K. B. MCCREDIE, G. P. BODEY, V. RODRIGUEZ and E. J. FREIREICH, Lymphocyte blastogenesis to human leukemia cells and their relationship to serum factors, immunocompetence and prognosis. *Cancer Res.* **32,** 2524 (1972).
29. I. HELLSTRÖM, H. O. SJOGREN, G. WARNER and K. E. HELLSTRÖM, Blocking of cell-mediated tumour immunity by sera from patients with growing neoplasms. *Int. J. Cancer* **7,** 226 (1971).
30. R. W. BALDWIN, M. H. EMBLETON and R. A. ROBINS, Cellular and humoral immunity to rate hepatoma-specific antigens correlated with tumour status. *Int. J. Cancer* **11,** 1 (1973).
31. F. VANKY, J. STJERNSWÄRD, G. KLEIN, L. STEINER and L. LINDBERG, Tumour-associated specificity of serum-mediated inhibition of lymphocyte stimulation by autochthonous human tumours. *J. nat. Cancer Inst.* **51,** 25 (1973).
32. J. INAGAKI, V. RODRIGUEZ and G. P. BODEY, Causes of death in cancer patients. *Cancer* **33,** 568 (1974).

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Clinical Trial of Clomiphene in Advanced Breast Cancer*

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Abstract—*The effect of clomiphene citrate was investigated in fifty postmenopausal women and one man with advanced breast cancer.*

Objective remission was observed in 20 *patients* (39·2%); 13 *out of* 31 *non-responders showed either arrest or objective regression in some metastases, while other deposits continued in progression.*

Side effects were mild and no patients required discontinuation of therapy for this reason; nausea was the most common side effect.

INTRODUCTION

IT HAS BEEN demonstrated recently that non steroidal agents, generally known as anti-estrogens, are able to reduce the incidence and size of dimethylbenz(a)anthracene (DMBA)-induced mammary carcinoma of the rat, [1, 2] as well as to compete *in vitro* with labelled estradiol for the estradiol binding protein (EBP) found either in the citoplasm of estrogen target cells or in breast tumor cells [1, 3–6].

Based on this experimental data, it seemed reasonable to try these agents in the treatment of advanced human breast cancer. Although a short experience with MER-25 [7] and with clomiphene citrate [8] was reported several years ago, it is only recently that the first statistical results of the clinical trials with anti-estrogens like nafoxidine [9] and tamoxifen [10, 11] have been published, with objective remission rates of 35 % for the former and more than 60 % for the latter.

The purpose of the present study was to assess the effect of clomiphene citrate in advanced breast cancer patients.

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MATERIAL AND METHODS

Fifty one patients, fifty women and one man, ranging in age from 27 to 85 years, with a median age of 59 years, were admitted to clinical trial if they fulfilled the following criteria: (1) advanced breast cancer, stage IV according to the classification of the Union Internationale Contre le Cancer (UICC); (2) histological diagnosis of the disease; (3) post-menopausal status either natural or surgical, of at least one year, (except in two patients who showed progression of the disease within one year after oophorectomy); and (4) free of any specific cancer treatment—radiotherapy, hormone therapy or chemotherapy—for at least three months.

Clomiphene citrate was given orally at two dose levels: 200 mg daily (43 patients) or 300 mg daily (8 patients) for at least 60 days unless unequivocal progression of the disease ocurred.

Each patient had a clinical control every three weeks; and a complete bone survey and chest X-ray examination every six weeks.

Results were evaluated according to the criteria followed by the European Breast Cancer Group [12].

RESULTS

According to their responses to treatment, the patients were divided into two groups: responders (Group I-R) and non-responders (Group II-NR).

Group I. (*Table* 1)

Twenty patients, 19 women and 1 man out of fifty-one, (39·2 %), showed an objective remission of the tumor which lasted between 70–660 days, with a mean duration of 365 days.

Table 1. *Clinical trial with clomiphene citrate in advanced breast cancer*

	Years after menopause				
Dominant lesion	< 1	1–4	5–9	> 10	Total
Soft tissue	—	1/5	0/2	4/10	5/17
Osseous	0/1	3/5	1/4	5/13	9/23
Visceral	0/1	2/2	1/4	2/3	5/10
Total	0/2	6/12	2/10	11/26	19/50

The numbers in the Table give the relation between objectives remissions and total patients treated.

Patients with visceral metastases had an increase rate of remissions followed in frequency by those with osseous and soft tissues deposits. There was a noticeable trend toward a greater rate of remission in patient groups within 1–4 and more than 10 years after menopause. Patients with less than 1 year elapsing after oophorectomy did not respond and only a small number of patients within 5–9 years after menopause showed remission.

Group II (*NR*)

Thirty-one patients showed no response to treatment according to the criteria followed in evaluation of results. However, 2 subgroups could be recognized: (1) NRa-18 patients in whom obvious progression of the disease was observed during treatment, which was discontinued within 30–240 days (mean 72 days). It should be remarked that the patient kept on treatment for as long as 240 days exhibited skin deposits growing very slowly until nearly the end of the therapeutical period when rapid tumor progression was observed. (2) NRb-13 patients showed partial response evidenced either by arrest or objective remission of growing metastases in some areas with simultaneous progression of other lesions; they were treated for periods of time ranging between 60 and 390 days (mean 150 days).

Effect of prior oophorectomy: 3 out of 4 patients who displayed objective remission to a previous oophorectomy again showed tumor regression with clomiphene citrate. On the other hand, one patient did not benefit from either oophorectomy or clomiphene.

Side effects were mild and no patients required discontinuation of therapy. Nausea was the most common symptomatic side effect; it was experienced by 12 patients (23·5 %) and was easily overcome with metoclopramide. Tinnitus was observed in 2 patients (4 %), blurred vision in 3 (6 %) and loss of equilibrium in 2 (4 %).

DISCUSSION

The results of this study indicate that clomiphene citrate exerts a therapeutic action in advanced breast cancer, inducing objective remission in 39 % of the patients treated. The results are comparable to those obtained by adrenalectomy or hypophysectomy [13–15] and on the same level as those achieved with nafoxidine [8] or tamoxifen [10].

The mean length of remission in our study was 12 months but it should be noted that some patients have enjoyed complete functional improvement for as long as two years at the time of writing this paper.

Three out of 4 patients who responded to a prior oophorectomy displayed a subsequent objective response to clomiphene citrate, comparable to what could be expected from a major endocrine ablation [13, 14].

No difference in response was observed among patients treated either with 200 or 300 mg of clomiphene daily. Side effects were mild and no patient required discontinuation of therapy for this reason.

It has been demonstrated that clomiphene citrate, like other anti-estrogen compounds is able to blockade *in vitro* the uptake of labelled estradiol by EBP [1, 3–5]. It was also shown that its administration reduced the number and size of DMBA-induced mammary carcinoma of the rat [1, 2]. It has been reported more recently, that nafoxidine causes a long term retention of the receptor–nafoxidine complex by rat uterine nuclei [16].

Therefore, it is tempting to speculate that clomiphene, like nafoxidine or tamoxifen, acts through its binding to EBP either by competing with estradiol at the same binding sites or by altering the properties of the receptor–ligand complex.

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REFERENCES

1. L. Terenius, Anti-oestrogens and breast cancer. *Europ. J. Cancer* **7,** 57 (1971).
2. J. C. Heuson, C. Waelbroeck, N. Legros, G. Gallez, C. Robyn and M. L. Hermite, Inhibition of DMBA-induced mammary carcinogenesis in the rat by 2-Br-ergocryptine (CB 154), and inhibitor of prolactin secretion, and by rafoxidine (U-11, 100 A) an estrogen antagonist. *Gynec. Invest.* **2,** 130 (1971/72).
3. B. G. Mobbs, The uptake of tritiated oestradiol by dimethylbenzoanthracene-induced mammary tumours of the rat. *Endocrinology* **36,** 409 (1966).
4. E. V. Jensen, G. E. Block, S. Smith, K. Kyser and E. R. De Sombre, Estrogens receptors and breast cancer response to adrenalectomy. *Nat. Cancer Inst. Monogr.* **34,** 55 (1971).
5. R. Hahnel, E. Twaddle and A. V. Vivian, Estrogen receptors in human breast. *In vitro* binding of estradiol by benign and malignant tumors, *Steroids* **18,** 681 (1971).
6. C. Geynet, C. Millet, H. Truong and E. E. Baulieu, Estrogens and anti-estrogens. Hormones and antagonist. *Gynec. Invest.* **3,** 2 (1972).
7. R. W. Kisiner and O. W. Smith, Observations on the use of a non-steroidal estrogen antagonist: MER-25. *Surg. Forum* **10,** 725 (1960).
8. A. L. Herbst, C. T. Griffiths and R. W. Kistner, Clomiphene citrate (NSC-35770) in disseminated mammary carcinoma. *Cancer Chemother. Rep.* **43,** 39 (1964).
9. E.O.R.T.C. Breast Cancer Group, Clinical trial of nafoxidine, an ostrogen antagonist in advanced breast cancer. *Europ. J. Cancer* **8,** 387 (1972).
10. M. P. Cole, C. T. A. Jones and I. D. H. Todd, A new antiestrogenic agent in late breast cancer. *Brit. J. Cancer* **25,** 270 (1971).
11. H. C. Ward, Anti-estrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. *Brit. med. J.* **1,** 13 (1973).
12. G.E.C.A., Protocole pour les essais cliniques de traitment des cancer mammaires en phase avancée. *Europ. J. Cancer* **2,** 201 (1967).
13. S. G. Taylor, Endocrine ablation in disseminated mammary carcinoma. *Surg. Gynec. Obstet.* **115,** 443 (1962).
14. J. B. Kennedy, Hormone therapy for advanced breast cancer. *Cancer* **18,** 1551 (1965).
15. H. Atkins, R. D. Bulbrock, M. A. Falconer, J. L. Hayward, K. C. MacLean and P. H. Schurr, Urinary steroids in the prediction of response to adrenalectomy or hypophysectomy. A second clinical trial. *Lancet* **2,** 1261 (1968).
16. J. H. Clark, J. N. Anderson and E. J. Peck, Estrogen receptor antiestrogen complex: atypical binding by uterine nuclei and effects on uterine growth. *Steroids* **22,** 707 (1973).

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The Influence of a Glucocorticoid on the Lodgement and Development in the Lungs of Intravenously Injected Tumour Cells*

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Abstract—*The enhancing effect of glucocorticoids on experimental metastasis formation, after intravenous injection of tumor cells, was investigated. Our results favour the hypothesis that the treatment diminishes the death rate of the injected cells during the initial period after arrival in the lungs. A possible explanation resides in the deleterious effect of glucocorticoids on the non-specific defence mechanisms of the host.*

INTRODUCTION

THE ENHANCING effect of glucocorticoids on metastasis in experimental animals [1–5] as well as in humans [6] is well documented. Most probably these drugs exert this promoting effect by increasing the "take" of circulating tumour cells [2–5]. Recently, the use of tissue cultured cells, labelled with radioactive markers [5, 7], has provided arguments that an increase in initial arrest of tumour cell emboli is responsible for the increase in the number of metastases. It remains controversial, however, whether this is the sole or even the most important factor. Indeed, the greater initial trapping of injected cells does not correlate very well, quantitatively, with the final number of metastases. On the other hand, it is well known that only very few tumour cells that become lodged in the lungs after intravenous injection are able to produce a metastatic lesion [2, 4, 8]. This is corroborated by the observation that most tumour emboli die rapidly, leaving only 1·5 % viable tumour cells 24 hr after injection [7]. This suggests that the initial survival rate of lodged tumour cells is of the utmost importance. Our experiments aimed at checking the hypothesis that the enhancing effect of glucocorticoids is produced largely during this phase of metastasis formation.

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MATERIAL AND METHODS

Cell culture

The MO_4 cells used were a gift from Dr. A. Billiau (Rega Institute, Louvain, Belgium), who described their origin previously [9]. A brief description will be given only. The MO_4 cell line was derived from a 3T3 cell line (C3H mouse embryo) by transformation *in vitro* with the Kirsten strain of MuSV. These cells produce invasively growing tumours of the fibrosarcomatous type in the syngenetic host (inbred C3H mice) at the site of inoculation. Spontaneous haematogenous metastasis

never occurs but progressively growing nodules are produced in the lungs after intravenous injection. The cells are cultured in EAGLE'S minimal essential medium supplemented with non-essential amino acids and 10% foetal bovine serum. They are routinely passaged by short trypsinization.

Animals

Female C3H mice with an average weight of 22 g were utilized for the *in vivo* experiments.

Experimental procedures and treatment

Experimental lung "metastases" were produced as follows: the cells were harvested by trypsinization and collection in complete growth medium, followed by one washing in medium without serum. During the inoculation the cells were kept at 4°C. Each mouse received 10^5 cells injected into the lateral tail vein in a volume of 0·2 cm^3. For labelling with sodium chromate (^{51}Cr, 200 mCi/mg; SCK Mol, Belgium) the cells were kept in suspension (containing 100 μCi/ml sodium chromate) at 37°C by agitation for 30 min. Hereafter the cells were washed 5 times with medium in order to remove the non-bound radioactivity. The mice were killed by ether overdosage and the appropriate organs were excised immediately. Radioactivity was determined in a Packard well-type gamma counter.

For autoradiographic localization of injected cells, actively growing cultures were labelled with 1 μCi/ml tritiated thymidine (^{3}HTdr, Amersham, 20·000 mCi/mmol) for 24 hr. Autoradiography showed that almost 100% of the cells were heavily labelled by this procedure. Before injection, the cells were washed 4 times with medium. The lungs were fixed in 10% paraformaldehyde and further processed for routine histology. Autoradiography was performed on 6 μm thick paraffin sections stained with PAS and counterstained through the emulsion with Carazzi's haematoxylin. The developed slides were observed with the Leitz Ultropack system with reflected light.

Mice were treated intraperitoneally either with 25 mg/kg triamcinolone acetonide (Kenalog®, Squibb), or with the solvent, in a volume of 0·2 cm^3, 4 hr prior to tumour cell injection.

RESULTS

Figure 1 shows the effect of triamcinolone acetonide on the number of pleural metastases 14 days after intravenous injection of 10^5 cells. The majority of the control animals have less than 10 nodules per lung in contrast to the treated animals where the number is too large to make a numerical evaluation possible. The size of the individual nodules, however, is largely the same in both groups. This made it possible to use the dry weight of the lungs as a quantitative measure for the number of metastases. This was determined after heating the excised lungs for 2 hr at 60°C. The results are given in Fig. 2. Apparently there exists a good correlation with the visual observation.

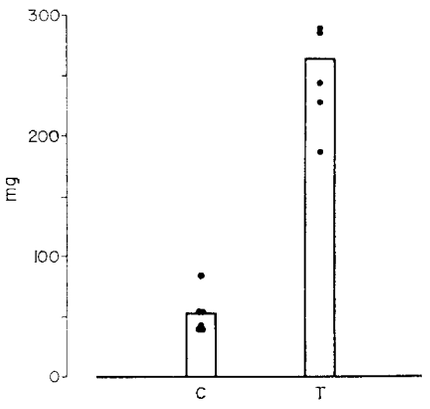


Fig. 2. Dry weight of the lungs 14 days after tumour cell injection (same animals as for Fig. 1). C = control animals; T = treated animals.

The next step was to determine the effect of triamcinolone treatment on trapping of labelled cells in the lungs. This was done in two separate experiments in order to confirm the relatively small effects. The distribution of label in different organs is shown in Fig. 3. It is obvious that most of the radioactivity is recovered in the lungs. Whether the label in the other organs is associated with viable cells is uncertain since no metastases develop in these organs. Triamcinolone pre-treatment consistently produced a redistribution of label: more being recovered in the lungs and less in spleen and liver. The augmented recovery in the lungs overwhelmed the diminution in liver and spleen since the total recovery in spleen, liver and lungs was enhanced too. The difference in label recovered in the lungs is about 20% only.

Since glucocorticoids can change the viability of many cells, both *in vivo* and *in vitro* [10] we checked whether there was any effect on the cells used for injection. The viability of these cells (assessed by phase-contrast observation) when cultured as a monolayer is not altered by cortisol. However, in order to imitate the *in vivo* situation, we incubated the cells with cortisol under continuous agitation which keeps the cells artificially in suspension. The viability was checked by the chromate release

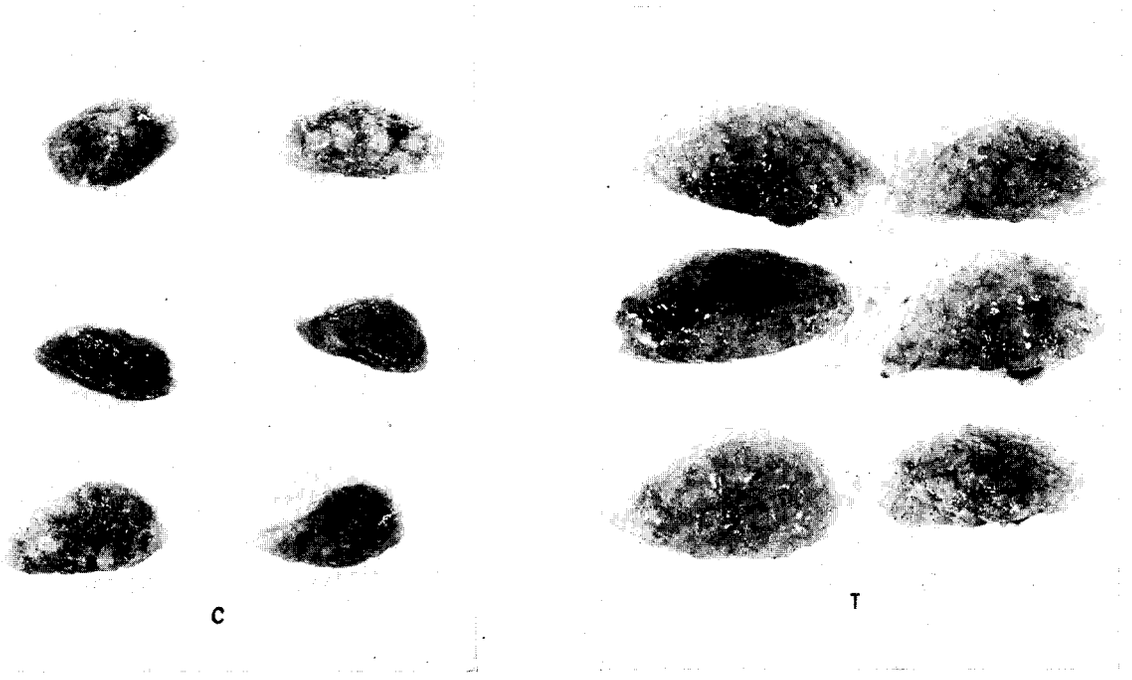


Fig. 1. *The left lungs of* 6 *control animals* (*C*) *and* 6 *treated animals* (*T*), 14 *days after injection of the cells. All animals had received* 10^5 MO_4 *cells into the lateral tail vein. The animals of group T were treated with* 25 *mpk triamcinolone acetonide,* 4 *hr prior to inoculation.*

(to face p. 752)

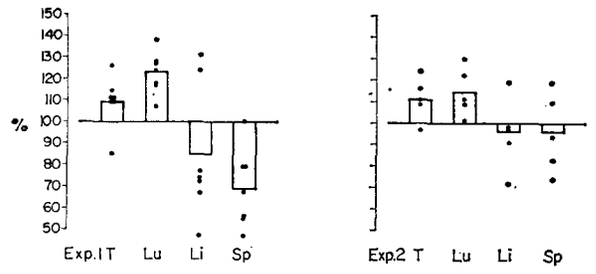


Fig. 3. *The effect of triamcinolone acetonide pre-treatment* (25 *mpk; 4 hr prior to tumour cell injection*) *on the distribution of radioactivity* 15 *min after injection of* 10^5 *sodium chromate labelled* MO_4 *cells into the lateral tail vein. The experiments were done with groups of paired animals* (1 *control animal and one treated animal*) *in order to keep pace with a very rigorous time schedule. The results are expressed as percentages of the control values. The distribution of label in the organs of the control animals was as follows: Experiment* 1: *liver* 25·5%; *spleen* 3%; *lungs* 71·5%. *Experiment* 2: *liver* 19%; *spleen* 1%; *lungs* 80%.

T = *total radioactivity in liver, spleen and lungs.*

Lu = *radioactivity in the lungs.*

Li = *radioactivity in the liver.*

Sp = *radioactivity in the spleen.*

method [11]. Here, no effect could be shown either (Fig. 4).

Finally, we wanted to check the distribution of injected cells with a morphological method since there was a rather poor correlation between the trapping of chromate labelled cells and the final number of metastases. For this purpose, we used cells labelled in the nucleus with tritiated thymidine. The distribution of these cells in the lungs was assessed with an autoradiographic technique. Since this method is very tedious and time consuming, only a limited number of animals could be used. Accordingly, the sampling technique was rigorously standardized. Histological 6 μm-thick sections were made parallel to the longitudinal axis of the lungs at the same plane in all specimens. After autoradiographic processing, these sections were scanned completely and the number of labelled nuclei per unit surface area was recorded. With this technique the only cells recorded are those that show an intact nucleus covered with developed silver grains. The surface was determined by planimetry of enlarged drawings of the slides. The results are shown in Table 1. The number of labelled cells declines rapidly, in both the treated and the control groups, with approximately one order of magnitude. The number of intact cells recovered in the treated group, however, is approximately 2 times larger after 2.30 hr and 4 times after 17.30 hr.

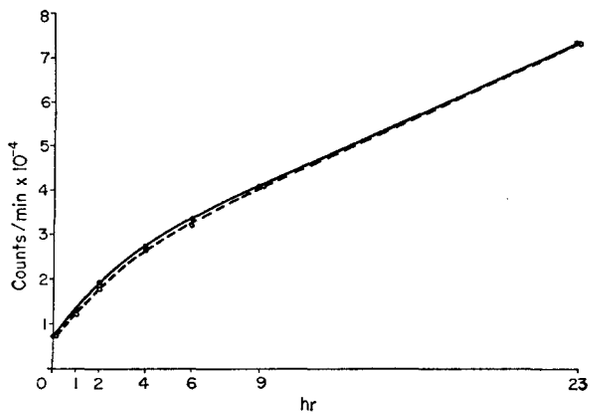


Fig. 4. *The cumulative release of radioactivity by* MO_4 *cells, labelled with sodium chromate* (^{51}Cr) *in suspension culture at* 37°C. *The treated culture contained* $10^{-6}M$ *cortisol. The culture contained* 10^6 *cells per ml in a total volume of* 25 *ml. A* 2 *ml sample of the supernatant was withdrawn at the indicated time intervals. The samples were centrifuged at* 3000 *rev/min in order to remove residual cells or debris. The radioactivity remaining in the supernatant was counted in a well type gamma counter.* (○——○ *control;* ○- - - -○ *treated*).

Table 1. *The effect of triamcinolone acetonide pre-treatment* (25 *mpk; 4 hr prior to tumour cell injection*) *on the number of labelled cells* (^3HTdr) *in the lungs. The animals were sacrificed* 2.30 *hours and* 17.30 *hours after tumour cell injection respectively. Two animals per group were used. The total number of sections scanned was* 52 *for each group*

Treatment group	Total no. of labelled cells/group	Number of labelled cells per cm²	Percentage of control value
Control 2.30 hr	146	1·47	
Treated 2.30 hr	250	3·38	230
Control 17.30 hr	18	0·12	
Treated 17.30 hr	37	0·44	366

DISCUSSION

The distribution and retention of tumour cells in different organs has been studied with several techniques [7, 12]. The use of cells labelled with sodium chromate has repeatedly been discussed as being unreliable because of reutilization of released label [7]. Whence a more stable labelling procedure has been proposed using iododeoxyuridine (^{125}I) incorporated into the DNA of the injected cells [7]. Another technique consists of a combination of labelled cells and morphological observation after radioautographic processing. We believe that the discrepancies in results obtained with these

different techniques are not due to the unreliability of some of them, but to the fact that each technique measures a different phenomenon. Indeed, the use of ^{3}HTdr labelled cells combined with autoradiography measures only those cells that still have an intact nucleus. Scintillation counting of ^{125}IUDR after injection of labelled cells measures, in addition, partly disintegrated cells and cells or DNA fragments taken up by phagocytes. The chromate technique measures viable cells and eventually some reutilized label. However, sodium chromate is released much more rapidly than IUDR from the cells when the viability declines. Since it is known that the viability of tumour cells lodged in the lungs declines at a very fast rate during the first minutes and hours after injection [7, 13], it can be expected that results obtained with the chromate technique should differ from other techniques. Moreover, this also could explain some lack of reproducibility between different experiments. The use of ^{3}HTdr labelled cells and autoradiography permits a clear distinction between intact cells and label retained in another form.

However, this rather time consuming technique does not facilitate a quantitative approach. If the foregoing remarks are kept in mind, we think that our results agree fairly well with those described by others [3–5], and give sufficient validity to the following comments. Obviously, the enhancing effect of glucocorticoids on metastasis is exerted during the initial period following the arrival of the cells in the lungs. This has already been shown very clearly by Albert [3]. Whence a growth promoting effect on the cells as a possible mechanism can be excluded. Indeed, only the number and not the size of the individual tumour nodules is increased. Three main modes of action by which glucocorticoids could exert their enhancing effect are possible:

(1) Alteration of the intrinsic viability of the cells.
(2) Alteration of the "recipient". This includes interference with the immunologic or non-specific defence mechanisms, or with the status of the recipient organ (e.g. vasculature).
(3) Alteration of the number of cells trapped initially in the lungs.

The third mechanism recently has been claimed to be the major cause of glucocorticoid induced enhancement of metastasis. However, several observations are in contradiction with this hypothesis. The majority of the injected tumour cells are trapped in the lungs during the first few minutes after injection, recirculation being a minor phenomenon [7]. If one supposes that the viability is not altered to a major extent by the treatment, then one should expect a quantitative correlation between the number of cells intially arrested in the lungs and the final number of metastatic nodules. However, Fidler [5] reported an enhanced trapping of IUDR labelled cells reacing 135 % of the control value while the final number of metastatic nodules was 593 % of the control value, which is in rather good agreement with our own results. Obviously, the potential of the cells to produce a metastic nodule must have been altered by the treatment after the trapping of the cells had been accomplished.

The survival of injected tumour cells repeatedly has been shown to decline dramatically during the first minutes and hours after inoculation [7, 13]. If the effect of glucocorticoids would be to slow down this rate of death, then one would expect the difference in recovered viable cells to become greater with time. Our results and those of others [5] show that this is indeed the case. Unfortunately, more prolonged observations are not totally reliable for different reasons (e.g., unstability of label, reutilization of label, dilution of label below a measurable threshold).

Although all arguments favour the hypothesis that the major effect of glucocorticoids is a result of a diminished rate of cell death, a minor contribution of enhanced trapping in the lungs should not be excluded.

Most probably the increased survival is not due to a direct effect on the injected cells (see *in vitro* results). Yet this cannot be excluded with certainty. Glucocorticoids are known to stabilize the cell membrane and to make cultured cells more resistant to several kinds of stress [10]. Inhibition of an immunological attack of the host is very unlikely to play a major role since this tumour is not immunogenic in the syngenetic animal (C3H mice). Indeed, immunosuppression, with cyclophosphamid (200 mpk) 5 days before cell inoculation does not enhance tumour growth. Moreover, attempts at immunization with a growing tumour followed by resection after 14 days and challenge after another 7 days are completely ineffective in terms of protection or influence on tumour growth (unpublished results). It has also been shown [3] that the idea of interference with specific immune mechanisms is hardly compatible with the rather narrow time space during which the animals must be treated in order to see any effect.

We favour the concept that glucocorticoids

depress the non-specific defence mechanisms that have been shown by many authors to exert a deleterious influence on neoplastic cells, regardless of their antigenicity. Indeed, the lytic action of non-immune lymphocytes and macrophages on the neoplastic cell has been claimed to be one of the major ways of defence against small foci of tumour cells [14, 15], and it is very well known that the activity of these cells is strongly affected by glucocorticoids [10, 16].

REFERENCES

1. M. Agosin, R. Christen, O. Badinez, G. Gasic, A. Neghme, O. Pizarro and A. Jarpa, Cortisone-induced metastases of adenocarcinoma in mice. *Proc. Soc. exp. Biol.* **80,** 128 (1952).
2. R. Baserga, P. Shubik and J. Baum, Action of cortisone on disseminated tumor cells after removal of the primary growth. *Science* **121,** 100 (1955).
3. D. Albert and I. Zeidman, Relation of glucocorticoid activity of steroids to number of metastases. *Cancer Res.* **22,** 1297 (1962).
4. I. Zeidman, The fate of circulating tumor cells. II. A mechanism of cortisone action in increasing metastases. *Cancer Res.* **22,** 501 (1962).
5. I. J. Fidler and S. Lieber, Quantitative analysis of the mechanism of glucocorticoid enhancement of experimental metastases. *Res. Commun. chem. Path. Pharmacol.* **4,** 607 (1972).
6. AMA Subcommittee, Meeting of the subcommittee on steroids and cancer. *J. Amer. med. Ass.* **146,** 655 (1951).
7. I. J. Fidler, Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with ^{125}I-Iodo-2′-deoxyuridine. *J. nat. Cancer Inst.* **45,** 773 (1970).
8. H. S. N. Greene and E. K. Harvey, The relationship between the dissemination of tumor cells and the distribution of metastases. *Cancer Res.* **24,** 799 (1964).
9. A. Billiau, H. Sobis, H. Eyssen and H. Van den Berghe, Non-infectious intracisternal A-type particles in a sarcome-positive leukemia-negative mouse cell line transformed by murine sarcoma virus (MSV). *Arch. ges. Virusforsch.* **43,** 345 (1973).
10. M. Dawson, *Cellular Pharmacology*, p. 187. Charles C. Thomas, Springfield, Ill. (1972).
11. J. C. Cerottini and K. T. Brunner, *In vitro* assay of target cell lysis by sensitized lymphocytes, In *In vitro Methods in Cell Mediated Immunity*. (Edited by B. R. Bloom and P. R. Glade), p. 369. Academic Press, New York (1971).
12. B. Fisher and E. R. Fisher, The organ distribution of disseminated ^{51}Cr-labeled tumor cells. *Cancer Res.* **27,** 412 (1967).
13. H. R. Withers and L. Milas, Influence of preirradiation of lung on development of artificial pulmonary metastases of fibrosarcoma in mice. *Cancer Res.* **33,** 1931 (1973).
14. J. B. Hibbs, L. H. Lambert and J. S. Remington, *In vitro* non-immunologic destruction of cells with abnormal growth characteristics by adjuvant activated macrophages. *Proc. Soc. exp. Biol.* **139,** 1049 (1972).
15. R. Keller, Cytostatic elimination of syngeneic rat tumor cells *in vitro* by non-specifically activated macrophages. *J. exp. Med.* **138,** 625 (1973).
16. W. L. Westor, M. J. Nandel, G. G. Krueger and M. D. Claman, Differential suppressive effect of hydrocortisone on lymphocytes and mononuclear macrophages in delayed hypersensitivity of guinea-pigs. *J. invest. Derm.* **59,** 345 (1972).

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Estrogen-binding *In Vitro* by DMBA-induced Rat Mammary Tumors: its Relationship to Hormone Responsiveness*

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Abstract—*Estradiol binding* in vitro *by a cytoplasmic fraction of DMBA-induced rat mammary tumors was related to a tumor responsiveness index (R.I.) which was based on the percentage loss of tumor volume for two weeks after ovariectomy. Binding affinity for estradiol in adenocarcinomas was similar to that found in previous work on these tumors and for human breast carcinomas. Estradiol binding by four fibroadenomas was very low. The correlation between estradiol binding and responsiveness was good in* 67% *of the adenocarcinomas. Possible reasons for lack of correlation in the remaining tumors are discussed.*

INTRODUCTION

IN RECENT YEARS, there have been numerous studies on the *in vitro* binding of estradiol by human breast cancer, and such assays are becoming of value in predicting the responsiveness of breast tumors to endocrine manipulation. One of the problems in setting up such an investigation is that full evaluation of the technique used is only possible after follow-up information has become available, so that the degree of correlation between the response of the patients to treatment and the results of the *in vitro* assay can be ascertained. For this reason, it has so far been possible to assess the accuracy of prediction of the assay used in only four studies [1–4]. We therefore decided to carry out preliminary evaluation of our assay for the measurement of the *in vitro* binding of estradiol by a "cytosol" fraction of human breast cancer specimens using the dimethylbenzanthracene (DMBA) induced rat mammary tumor. It has been established both by *in vivo* [5, 6] and *in vitro* [2, 7] methods that estradiol binding by this tumor is related to its responsiveness to ovariectomy. Tumors which regress after ovariectomy have been shown to bind estradiol to a greater extent than those which continue to grow. The hormone responsive tumors possess high-affinity estrogen binding sites which are very similar to those found in some human carcinomas [8]. However, these tumors show great variability in their degree of responsiveness to ovariectomy, and we report here an attempt to relate the degree of hormone responsiveness to the amount of estradiol bound by a cytoplasmic fraction of the tumors. A similar preliminary investigation on a small number of tumors has been reported recently by Nomura *et al.* [9], who obtained a good correlation between estradiol binding and responsiveness to ovariectomy.

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MATERIAL AND METHODS

Reagents

Estradiol [6, 7—^{3}H] (specific activity 40–50 Ci/mmole) was obtained from New England Nuclear Corporation. On arrival, this was diluted to 10μCi/ml redistilled benzene:ethanol (9:1). Aliquots of this stock solution were evaporated under nitrogen immediately before the tumor assay. 7,12-DMBA and unlabelled estradiol-17β were obtained from Sigma Chemical Co., St. Louis, Miss.; dextran T 70 from Pharmacia, Montreal and charcoal (Norit A) from Matheson, Coleman and Bell.

The scintillator used was 5 gm PPO & 0·1 gm POPOP (Amersham-Searle) per 1. toluene.

Animals—induction of tumors and evaluation of hormone responsiveness

Mammary tumors were induced in female Holtzman rats (Holtzman, Wisconsin, U.S.A.) by the gastric instillation of DMBA in corn oil (10 mg/ml). An initial dose of 15 mg was given at age 50–52 days, and a second dose of 10 mg one week later. Tumors began to appear 4–5 weeks after the second dose. They were measured twice weekly through the skin with calipers, along the largest diameter and then at right angles to it across the broadest part of the tumor. When the mean diameter of a tumor was approximately 2·0 cm, the animal bearing it was ovariectomized and measurement was continued until the response of the tumor was ascertained. To ensure that regression was due to the removal of endogenous hormone, and was not spontaneous, tumors were restimulated by injecting the animals three times weekly with 2 μg estradiol with 8 mg progesterone in 0·4 ml corn oil s.c. until regrowth was established. Regressed tumors which did not grow during this treatment were not used. So that all animals would have comparable hormonal status at the time of sacrifice, animals bearing unresponsive tumors were also given at least one estradiol + progesterone injection. Injections were stopped at least four days before the tumors were used for assay to free estrogen-binding sites of injected hormone.

As mentioned above, there is considerable variation in the responsiveness of these tumors to ovariectomy and hormonal replacement. Therefore, the time between ovariectomy and sacrifice varied from 20 to 58 days. Occasionally, tumors which regressed initially after ovariectomy began to grow again before hormonal replacement was begun. Presumably regrowth was due to the independent cells present in the total cell population. The degree of responsiveness of all the tumors was evaluated by the calculation of a "responsiveness index" (R.I.) based on the change of tumor volume which occurred 11–15 days after ovariectomy. The formula of a prolate ellipsoid (length × width^2/2) was used to calculate tumor volume [10]. The R.I. is defined as the mean change in volume (expressed as a percentage of the volume at ovariectomy) per day, measured over 11–15 days after ovariectomy. Tumors which regressed after ovariectomy had a positive R.I., while tumors which continued to grow had a negative R.I. Tumors which remained static had an R.I. of 0.

Animals were killed by decapitation, and the tumor(s) was (were) removed. Cystic and necrotic areas were cut away and discarded. A sample of each tumor was taken for histological examination. Tumors were either homogenized immediately or stored at −17°C for up to four weeks (but usually less than 1 week). Estradiol-binding assays were also carried out on uterus and muscle (diaphragm) from rats weighing approximately 200 g., which had been ovariectomized two days previously.

Estradiol-binding assay

The method used was derived from those of Korenman and Dukes, Feherty *et al.* and Hähnel and Twaddle [11–13]. Tissues were homogenized in Tris–HCl buffer (pH 8·0) containing 1·5 mM EDTA, the final concentration of homogenate being 100 mg/ml buffer. Homogenization was carried out in an ice-cooled vessel with a Porter–Elvehjem type homogenizer using 15 sec bursts at 790 rev/min separated by 45 sec cooling periods. The homogenate was centrifuged at 48,000 **g** for 1 hr at 0°C. The supernatant was removed, and was either assayed immediately, or was stored at −17°C. Storage for 1 week at this temperature did not result in loss of binding activity. For assay, the supernatant was diluted 1:1 with buffer and replicate aliquots were incubated with 1·1 × 10^{-9}M [^{3}H]-estradiol. Unlabelled estradiol was added to some aliquots at a concentration of 4 × 10^{-7}M. Blanks containing 1·0 ml of buffer instead of "cytosol" were run concurrently. Incubation was carried out at 25°C for 30 min. The tubes were then cooled to 0°C in an ice-bath and 1·0 ml of a suspension of dextran–charcoal (0·05:0·5%) in the homogenization buffer was added. The tubes were shaken and allowed to stand at 0°C for 15 min. The charcoal was removed by centrifuging at 15,000 **g** for 15 min at 0°C. The supernatant was then recentrifuged to ensure complete removal of charcoal. Estradiol remaining in

the supernatant was extracted with 15 ml methylene chloride overnight at 4°C. After centrifuging at low speed for 10 min a 10 ml aliquot of the methylene chloride layer from each tube was removed to a counting vial and evaporated. Scintillator was added and radioactivity was measured in a Packard scintillation counter (Model 3375) at an efficiency of approximately 44%. Quenching was corrected for using an external standard. The difference in the amounts of radioactivity in the absence and in the presence of unlabelled estradiol was considered to be due to binding by the high-affinity estradiol receptor. This was expressed as specific binding per mg of tissue homogenized.

Scatchard plot analysis

One millilitre aliquots of "cytosol" were added to an equal volume of Tris–HCl buffer containing increasing amounts of [^{3}H-17β]-estradiol ranging in concentration from 2·67 $\times 10^{-10}$M to 4·28 $\times 10^{-9}$M. Non-specific binding was determined by the addition of unlabelled estradiol to give a concentration 400 times that of the labelled hormone (1·1 $\times 10^{-7}$M – 1·7 $\times 10^{-6}$M).

Removal of unbound estradiol by charcoal adsorption and determination of specifically bound estradiol were carried out as described above.

Bound estradiol was plotted against bound/free. The dissociation constant (*Kd*) of the estradiol–receptor complex was calculated from the slope of the plot and the concentration of binding sites by extrapolation of the graph to the abscissa [12, 14].

RESULTS

Twenty-four adenocarcinomas and four fibroadenomas were assayed for estradiol-binding capacity. The amount of radioactivity bound, related to the wet weight of tissue, and the R.I. for each tumor is presented in Table 1, and plotted in Fig. 1. Radioactivity bound by uterus and diaphragm is included in Table 1. All four fibroadenomas and one carcinoma bound less than the muscle per unit wet weight. The binding values for the remaining tumors range from 22·9 to 217·8 dis/min per mg of tissue. This ten-fold range is similar to that found by Terenius [15] using a similar technique, although his results are expressed per unit of cytosol protein. The highest binding tumor bound approximately one-tenth the amount of ^{3}H-estradiol bound by uterus. It is clear that both the R.I. and the estradiol-binding values form a continuum, and that

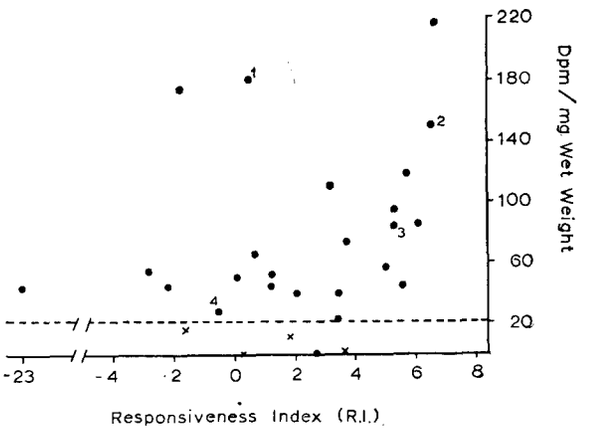


Fig. 1. Estradiol-binding values in DMBA-induced rat mammary carcinomas and fibroadenomas plotted against responsiveness index as defined in the text. ●, adenocarcinomas; ×, fibroadenomas. The numbers 1–4 indicate tumors used for Scatchard plot analysis (see Fig. 2). The horizontal dotted line indicates the amount of estradiol bound by diaphragm muscle from a rat weighing 185 g, ovariectomized two days before assay.

Table 1. Estrogen-binding capacity and responsiveness index (R.I.) for DMBA-induced rat mammary adenocarcinomas and fibroadenomas. F, fibroadenoma; indices 1–4 indicate tumors used for Scatchard plot analysis in Fig. 2. Uterus and muscle were taken from a rat weighing 185 g which had been ovariectomized two days before the assay; the values for estrogen binding capacity are representative of those obtained in several experiments.

Tissue or tumor no.	Dis/min per mg wet weight	R.I.
15	217·8	6·8
23[1]	180·6	0·7
7	175·0	– 1·6
21[2]	150·5	6·6
13	119·5	5·8
14	111·6	3·2
6	97·5	5·4
31	84·3	6·1
22[3]	83·9	5·4
9	74·4	3·8
27	66·3	0·8
29	58·0	5·2
10	55·8	– 2·8
19	52·8	1·2
24	51·6	0·2
8	46·8	–23·0
26	45·1	– 2·1
32	45·0	0·3
28	42·5	5·6
25	40·8	2·1
17	40·4	3·5
12[4]	28·6	– 0·5
20	22·9	4·0
33	1·6	2·7
5 F	16·4	– 1·6
30 F	10·3	1·9
3 F	1·5	3·6
1 F	0·6	0·3
Uterus	2075·4	
Muscle	20·3	

there was no sharp distinction between responsive and unresponsive tumors. There was a correlation between the R.I. and estradiol-binding capacity, but this was by no means perfect. Two of the 10 high-binding tumors had low R.I.'s (−1·6, 0·7) *i.e.*, they were "false positives". The relationship was less good for the low-binding tumors, only 8 of the 14 lowest binding adenocarcinomas having R.I.'s<1·5. Figure 2 shows Scatchard plots drawn from binding data from four tumors of varying response to ovariectomy. The corresponding tumors are indicated in Fig. 1. Three of the tumors had binding values >80 dis/min per mg. All three had similar *Kd's* (6·2–8·6 × 10^{-10}M). The most responsive of these four tumors

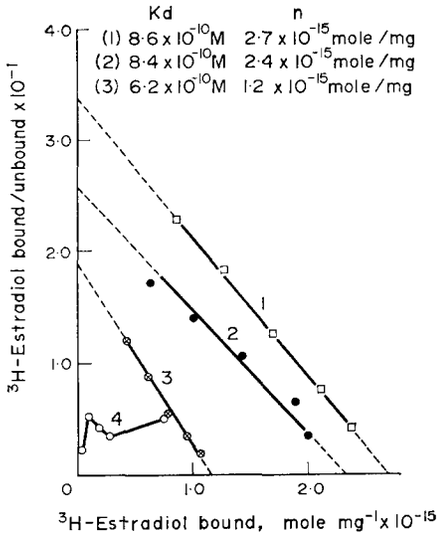


Fig. 2. Scatchard plot analysis of the four tumors indicated in Fig. 1. Kd, dissociation constant of estradiol–receptor complex; n, concentration of binding sites related to wet weight of tissue taken.

(R.I. 6·6) contained binding sites at a concentration of 2·4 × 10^{-15} moles/mg, twice the concentration of sites in the tumor with R.I. 5·4. The highest concentration of sites (2·7 × 10^{-15} moles/mg) was found in the "false positive" tumor (R.I. 0·66). The unresponsive tumor (R.I. −0·5) had no high-affinity binding sites. The *Kd* for binding in the uterus was 5·63 × 10^{-10}M and the concentration of binding sites 2·45 × 10^{-14} moles/mg tissue.

DISCUSSION

The tightness of binding of estradiol by cytoplasmic receptors (as expressed by the *Kd* values) in the DMBA-induced tumors and in uterus assayed by this method was similar to that found by most other authors for rat and human breast tumors [7, 8, 12, 16–21, and our unpublished results] and rat uterus [22], but approximately one hundred times less than that found for human breast tumors by Hähnel and Twaddle [23]. The concentration of receptor sites in ovariectomized rat uterus (expressed per mg of tissue) assayed by our method was considerably higher than that found in the same tissue by Feherty *et al.* [22]. This may be due to the fact that in our assay, the charcoal separation of bound and unbound estradiol was carried out at 0°C, whereas in that of Feherty *et al.*, it was carried out at 30°C. A degree of receptor lability at this temperature has been reported [24, 25].

It is likely that complete independence from hormonal control is uncommon in DMBA-induced rat mammary tumors. In only 2 tumors of the 28 investigated here did growth continue at the same or at a greater rate after ovariectomy. These 2 tumors bound estradiol to a greater extent than muscle.

If the results shown in Table 1 and Fig. 1 had been used "blind" to estimate the responsiveness of these experimental mammary carcinomas, using 70 dis/min per mg as an arbitrary "cut-off" point between high- and low-binding carcinomas, and an R.I. <1·5 as a criterion of unresponsiveness, a correct result would have been obtained for 16/24 tumors. In those binding >70 dis/min per mg tissue, the binding value was a good indication of the rate of regression after ovariectomy in 8/10 tumors. The low binding by fibroadenomas is in agreement with similar results in human benign breast disease [3, 12, 13, 16, 20, 26, 27, and our unpublished results]. However, the low-binding rat carcinomas showed a large variation in responsiveness, and the correct response would have been estimated from the binding values in only 8/14 low-binding tumors. There are a number of factors which may have contributed to lower binding values than would have been expected for the remaining 6 tumors, the most important of which was probably inaccurate tumor volume measurement. Although the formula for a prolate ellipsoid has been used as an approximation for tumor volume, those tumors are, in fact, quite variable in shape, and sometimes contain cystic and/or necrotic areas which contribute to inaccuracies in estimating the volume of viable tissue. Also owing to the variation in response to restimulation after ovariectomy, the time which elapsed between ovariectomy and the assay of estrogen-binding

capacity was sometimes several weeks, so that it is possible that the estrogen-binding capacity had changed since responsiveness had been estimated. It might be preferable to investigate the estradiol-binding capacity of biopsies of the tumors at the time of ovariectomy, rather than after the responsiveness has been determined. In these experiments, estradiol binding is expressed relative to the weight of tissue homogenized. As these tumors (except fibroadenomas) were all largely epithelial in nature, the amount of stroma present probably does not introduce a large error, but in future experiments the estradiol binding value will be related to the DNA concentration in the homogenate. "False negatives" have also been reported in *in vitro* studies in human tumors [1, 2], but none was reported in *in vivo* studies on the uptake of estrogen by DMBA-induced rat mammary tumors or by human breast tumors [28–30] or in an *in vitro* study on slices of DMBA-induced mammary tumors [6]. It is felt that data on the apparently low-binding, but responsive, tumors in the present study are the result of a combination of the factors mentioned above, and that a better correlation of responsiveness and estrogen binding could be obtained by greater standardization of timing concerning ovariectomy, restimulation and sacrifice, and possibly by relating binding values to DNA concentration rather than to wet weight.

However, the difference between the estrogen binding values for the two "false positive" tumors and the other tumors with similar R.I.'s is too great to be accounted for by inaccurate volume measurements and variations in cellularity. "False positives" have also been reported in studies of human tumors, both *in vitro* [1, 2, 4] and *in vivo* [30]. Some of the patients concerned had previously experienced remission after ovariectomy, or experienced subjective remission after endocrine ablation [1, 2]. No false positives were reported in the *in vivo* or slice studies on DMBA-induced rat tumors already mentioned [5, 6, 28].

There have been a number of recent reports concerning the binding of steroid hormones by a high-speed cytosol fraction of various tissues in which steroid binding accompanied by unresponsiveness to the hormone has occurred [31–37]. All these studies were carried out on tissues derived from organs which are normally hormone-responsive, but which presumably have undergone modification during ontogeny or pathogenesis. The modification appears to be related to a defect in the mechanism for the transfer of steroid from the cytoplasm to the nucleus. It is possible that a similar defect has occurred during the pathogenesis of the "false-positive" DMBA-induced tumors described here and of "false positive" human breast tumors described by others. If nuclear transfer is the critical factor in determining tumor responsiveness to hormones, it may be necessary to modify hormone-binding assays to achieve more complete correlation between assay and response.

REFERENCES

1. E. Engelsman, J. P. Persijn, C. B. Korsten and F. J. Cleton, Oestrogen receptor in human breast cancer tissue and response to endocrine therapy. *Brit. Med. J.* **2,** 750 (1973).
2. E. V. Jensen, G. E. Block, S. Smith, K. Kyser and E. R. DeSombre, Estrogen receptors and hormone dependency. In *Estrogen Target Tissues and Neoplasia* (Edited by T. L. Dao), p. 23. Univ. of Chicago Press (1972).
3. B. S. Leung, W. S. Fletcher, T. D. Lindell, D. C. Wood and W. W. Krippaehne, Predictability of response to endocrine ablation in advanced breast carcinoma. *Archs. Surg.* **106,** 515 (1973).
4. H. Maass, B. Engel, H. Hohmeister, F. Lehman and G. Trams, Estrogen receptors in human breast cancer tissue. *Amer. J. Obstet. Gynec.* **113,** 377 (1972).
5. B. G. Mobbs, The uptake of tritiated oestradiol by dimethyl-benzanthracene-induced mammary tumours of the rat. *J. Endocr.* **36,** 409 (1966).
6. S. Sander and A. Attramadal, The *in vivo* uptake of oestradiol-17β by hormone responsive and unresponsive breast tumours of the rat. *Acta path. microbiol. scand.* **74,** 169 (1968).
7. W. L. McGuire and J. A. Julian, Comparison of macromolecular binding of estradiol in hormone-dependent and hormone-independent rat mammary carcinoma. *Cancer Res.* **31,** 1440 (1971).
8. W. L. McGuire and M. DeLaGarza, Similarity of the estrogen receptor in human and rat mammary carcinoma. *J. clin. Endocr. Med.* **36,** 548 (1973).

9. Y. Nomura, Y. Abe, T. Hattori and K. Inokuchi, Measurement of estrogen-binding capacity in the hormone-dependent and -independent rat mammary tumors. *Gann* **64,** 401 (1973).
10. L. Simpson-Herren and D. P. Griswold, Studies of the cell population kinetics of induced and transplanted mammary adenocarcinoma in rats. *Cancer Res.* **33,** 2415 (1973).
11. S. G. Korenman and B. A. Dukes, Specific estrogen binding by the cytoplasm of human breast carcinoma. *J. clin. Endocr.* **30,** 639 (1970).
12. P. Feherty, G. Farrer-Brown and A. E. Kellie, Oestradiol receptors in carcinoma and benign disease of the breast. *Brit. J. Cancer* **25,** 697 (1971).
13. R. Hähnel, E. Twaddle and A. B. Vivian, Estrogen receptors in human breast cancer. 2. *In vitro* binding of estradiol by benign and malignant tumors. *Steroids* **18,** 681 (1971).
14. G. Scatchard, The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).
15. L. Terenius, Estrogen and progestogen binders in human and rat mammary carcinoma. *Europ. J. Cancer* **9,** 291 (1973).
16. S. G. Korenman, Human breast cancer as a steroid hormone target. In *Estrogen Target Tissues and Neoplasia* (Edited by T. L. Dao), p. 59, Univ. of Chicago Press (1972).
17. G. Leclerq, J. C. Heuson, R. Schoenfeld, W. H. Matteiem and H. J. Tagnon, Estrogen receptors in human breast cancer. *Europ. J. Cancer* **9,** 665 (1973).
18. G. Leclerq and J. C. Heuson, Specific estrogen receptors of the DMBA-induced mammary carcinoma of the rat and its estrogen-requiring molecular transformation. *Europ. J. Cancer* **9,** 675 (1973).
19. W. L. McGuire, Estrogen receptors in breast cancer. *J. clin. Invest.* **52,** 73 (1973).
20. W. L. McGuire, J. A. Julian and G. C. Chamness, A dissociation between ovarian dependent growth and estrogen sensitivity in mammary carcinoma. *Endocrinology* **89,** 969, (1971).
21. J. L. Wittliff, R. Hilf, W. F. Brooks, E. D. Savlov, T. C. Hall and R. A. Orlando, Specific estrogen-binding capacity of the cytoplasmic receptor in normal and neoplastic breast tissues of humans. *Cancer Res.* **32,** 1983 (1971).
22. P. Feherty, D. M. Robertson, H. B. Waynforth and A. E. Kellie, Changes in the concentration of high-affinity oestradiol receptors in rat uterine supernatent preparations during the oestrous cycle, psuedopregnancy, maturation and after ovariectomy. *Biochem. J.* **120,** 837 (1970).
23. R. Hähnel and E. Twaddle, Estimation of the association constant of the estrogen-receptor complex in human breast cancer. *Cancer Res.* **33,** 559 (1973).
24. H. Braunsberg, E. Killen and S. G. Richardson, The effect of thiols on oestradiol binding by components of human malignant breast tumours. *Biochem. J.* **130,** 38P (1972).
25. W. L. McGuire and M. DeLaGarza, Improved sensitivity in the measurement of estrogen receptor in human breast cancer. *J. clin. Endocr.* **37,** 986 (1973)
26. 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).
27. C. B. Korsten and J-P. Persijn, A simple assay for specific estrogen binding capacity in human mammary tumours. *Z. klin. Chem.* **10,** 502 (1972).
28. B. G. Mobbs, The uptake of simultaneously administered [^{3}H] oestradiol and [^{14}C] progesterone by dimethylbenzanthracene-induced rat mammary tumours. *J. Endocr.* **41,** 339 (1968).
29. P. J. Folca, R. F. Glascock and W. T. Irvine, Studies with tritium-labelled hexoestrol in advanced breast cancer. *Lancet* **2,** 796 (1961).
30. H. Braunsberg, V. H. T. James, W. T. Irvine, F. James, C. W. Jamieson, R. A. Sellwood, A. E. Carter, and M. Hulbert, Prognostic significance of oestrogen uptake by human breast cancer tissue. *Lancet* **1,** 163 (1973).
31. G. Shyamala, Estradiol receptors in mouse mammary tumors—absence of the transfer of bound estradiol from the cytoplasm to the nucleus. *Biochem. biophys. Res. Commun.* **46,** 1623 (1972).
32. J. D. Wilson and J. L. Goldstein, Evidence for increased cytoplasmic androgen binding in the submandibular gland of the mouse with testicular feminization. *J. biol. Chem.* **247,** 7342 (1972).

33. J. F. DUNN, J. L. GOLDSTEIN and J. D. WILSON, Development of increased cytoplasmic binding of androgen in the submandibular gland of the mouse with testicular feminization. *J. biol. Chem.* **248,** 7819 (1973).
34. E. M. RITZÉN, S. N. NAYFEH, F. S. FRENCH and P. A. ARONIN, Deficient nuclear uptake of testosterone in the androgen-insensitive (Stanley–Gumbreck) pseudohermaphrodite male rat. *Endocrinology* **91,** 116 (1972).
35. M. VÉRTES and R. J. B. KING, The mechanism of oestradiol binding in rat hypothalamus: effect of androgenization. *J. Endocr.* **51,** 271 (1971).
36. L. PLAPINGER and B. MCEWEN, Ontogeny of estradiol-binding sites in rat brain. I. Appearance of presumptive adult receptors in cytosol and nuclei. *Endocrinology* **93,** 1119 (1973).
37. L. PLAPINGER and B. MCEWEN, Ontogeny of extradiol-binding sites in rat brain. II. Characteristics of a neonatal binding macromolecule. *Endocrinology* **93,** 1129 (1973).

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Increased Growth-inhibiting Effect on Tumour Cells of Protamine after Polymerization

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Abstract—*Protamine sulphate was polymerized by peptide-bond formation using a water-soluble carbodiimide. The procedure yielded dimer and tetramer forms of protamine, without loss of positively charged arginyl groups. Binding of protamine and its dimer and tetramer forms to JB-1 ascites tumour cells was studied by cell electrophoresis. It was found that increasing binding affinity was obtained by increasing the molecular weight.*

The effect of these protamines on the growth of the JB-1 ascites tumour in AK/Aa mice was studied, and it was found that whereas monomer protamine had no effect on the development of the tumour, the tetramer, and to a lesser degree, the dimer forms of protamine inhibited tumour growth. It is suggested that the biological effect increases with increasing affinity of the protamines for the cell surface.

INTRODUCTION

THE NEGATIVE surface charge of animal cells has been well documented by means of cell electrophoresis [1–3], and chemical investigations have resulted in the identification of the negatively charged groups as acidic sugar residues in glycoproteins and glycolipids. Electrostatic interaction of the anionic sites of the cell surface and basic polymers [polycations (PC)] results in decreased negative surface charge density. This interaction usually is followed by numerous biological changes such as inhibition of growth agglutination, lysis and shrinking [3, 4].

The role of the chemical nature and chain length of PCs for interactions with cell surfaces was first noted by Katchalsky *et al.* [5], who demonstrated that the interaction of polylysine with red blood cells was augmented when the chain length of the PC was increased. Furthermore, PC-enhanced uptake of albumin into tumour cells exhibited increased effect with increasing mol wt of PC [6]. Also, the effect of various DEAE-dextrans with different molecular size and charge density on electrophoretic mobility and transplantability of 4 mouse tumours showed the same relationship between mol wt and biological effect.

Reilly and Shah [7] reported that polylysine and lysine-rich histones inhibited the growth of transplantable mouse mammary tumours, whereas arginine-rich histones and protamines (the latter being strong PCs containing 70–90 % arginine) caused little or no inhibition in spite of a great charge density. In the early 1960s, O'Meara and O'Halloran [8], showed that so-called "prolonthan" preparations, which are formaldehyde-treated protamine sulphate (PS), inhibit transplantable mouse tumour as well as human tumour growth. According to Fraenkel-Conrat and Olcott [9], formaldehyde binds to the guanidyl groups, a reaction which is accompanied by extensive cross-linking leading to polymerization and a decrease in the solubility of the protein. It has been claimed that the growth-retarding action of "prolon-

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than" was due to a slow liberation of protamine in the organism after injection [8].

This paper shows that an increase in the mol wt of PS by a controlled polymerization converts PS to a highly potent tumour-toxic PC. Reversibility of PC-cell surface interaction is shown by competitive inhibition with a strong polyanion, dextran sulphate (DS).

MATERIAL AND METHODS

Animals and tumour

The animals used were AK/Aa mice maintained at the Institute of Cancer Research, Aarhus. The mice, which were 2 months old and weighed 24–32 g at the beginning of the experiment, were divided into groups of 7–10 animals consisting of 50 % of each sex.

The tumour used throughout the experiment was the ascitic form of the JB-1 tumour (a plasma-cytoma described by Bichel [10].

Polymerization of protamine

Formaldehyde seemed unsuitable as condensing agent since the polymerization reaction is difficult to control. A water-soluble carbodiimide, viz. 1-ethyl-3 (3-dimethyl-amino-propyl) carbodiimide (CDI), Sigma Chemical Company, which is used in peptide synthesis, was chosen. The reaction between PS (a gift from Nordisk Insulin Laboratorium) and CDI was carried out in cold water. Buffered solutions were not observed to enhance polymerization.

Conditions of reaction: PS, 100 ml, was dissolved in 3·0 ml of glass-distilled water and cooled to 0°C in an ice bath. CDI, 1·0 g, in 2·0 ml water (0°C) was slowly added to the PS suspension. The mixture was allowed to react for 6–10 days in a refrigerator (4°C). Prolonged shaking of the mixture ruined the polymerization.

The reaction was stopped by fractionating the mixture on a column (2·5 × 35·0 cm) packed with Sephadex G-75 superfine. Elution was performed with 0·15 M NaCl. Flow rate: 14·6 ml/hr. Blue dextran (Pharmacia, Uppsala) was applied to the column to check bed packing and to determine the void volume. The elution profile of the PS derivatives was determined by the method of Lowry [11] on aliquots from the eluate, since PS exhibits no ultraviolet absorption and CDI is stained by this method (as well as the protein).

The peaks of modified PS (Fig. 1) were collected and concentrated using hollow-fibre dialysing technique against 15 % $CaCl_2$. Finally, the concentrates were dialysed against distilled cold water (4°C) and lyophilized.

Analysis

The amount of cationic groups in PS and PS derivatives was determined to ensure that the charge density was intact after reaction with CDI. This was done by measuring the amount of arginine in PS [12], the only charged group of protamine. The charge density in PS and the PS derivatives was compared by titration with DS (Pharmacia, Uppsala) [13].

Molecular weights of PS and CDI-treated PS were determined by thin-layer gel filtration by developing them together with reference proteins of known mol wt on the thin-layer plate.

Biological testing

An amount of 5–10 ml of JB-1 ascites fluid obtained from mice transplanted 6–10 days earlier was washed with 2 × 10 ml of saline, centrifuged at 50 × **g** for 10 min and diluted with saline to give a final concentration of 5×10^6 JB-1 cells/ml. One ml of 2 mg/ml PS, PS derivatives, or saline was incubated for 1 hr with 1×10^6 JB-1 cells at room temperature in disposable syringes. The contents were mixed carefully at short intervals during the incubation period and then injected into groups of 7–10 mouse. The protein solutions were made isotonic by adding NaCl. Subsequent treatment of the cells for 5 min with DS before injection was performed by introducing into the same syringes 0·5 ml of DS (4 mg/ml saline) solution followed by thorough mixing. The evaluation of the effect of tumour-cell incubation with the drugs was based on the observation of the average survival time of inoculated mice, Fig. 2.

Cell electrophoresis

Cellular electrophoretic mobilities were measured at 25°C ± 0·1° in a rectangular measuring cuvette fitted with platinum electrodes (Zeiss Zytopherometer).

Tumour cells and normal human erythrocytes were prepared for electrophoresis by washing them three times in phosphate-buffered saline (PBS), pH 7·4, and once in PBS–sorbitol, 1:4 (1 vol. PBS, 4 vol. sorbitol 5 %. Buffers were prepared from glass-distilled water. After resuspension in PBS–sorbitol, 1:4, the cells were kept at 0–2°C until use. The times for cells to traverse 31 or 124 μm in both directions at a current of 3·0 mA were recorded. In the standard procedure, 20 cells were measured in both directions in each concentration of PS and PS derivatives.

Tumour cells were taken from donor mice inoculated 6–10 days earlier, and human erythrocytes were used as a reference to check the cell-electrophoresis apparatus.

RESULTS

The elution profile of the reaction mixture is seen in Fig. 1. It will be noted that the polymerization product consists of two well-defined peaks, and that the PS derivatives are separated from CDI. The elution peak of unmodified PS is marked on the figure.

The results from the mol wt determinations on thin-layer gel filtration showed that the polymerization procedure used yields a dimer and a tetramer of the monomer PS (henceforth called di-PS and tetra-PS).

Determination of the amount of the only charged group in protamine, arginyl, shows a content of 83 g arginine/100 g protamine, and by means of turbidometric titration, it was confirmed that none of the cationic groups o

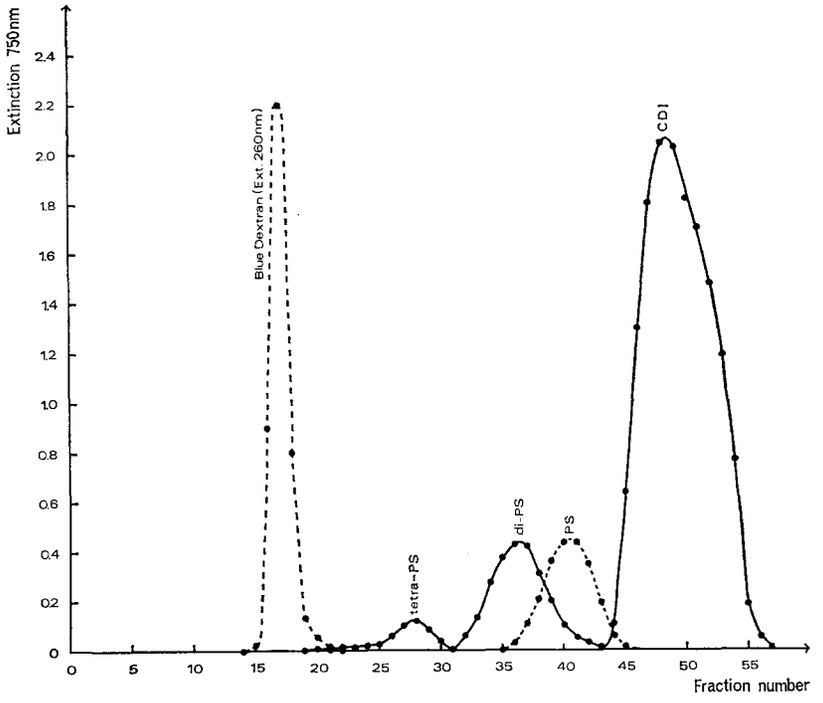


Fig. 1. Elution profile from a G-75 Sephadex column of the reaction mixture of polymerization.

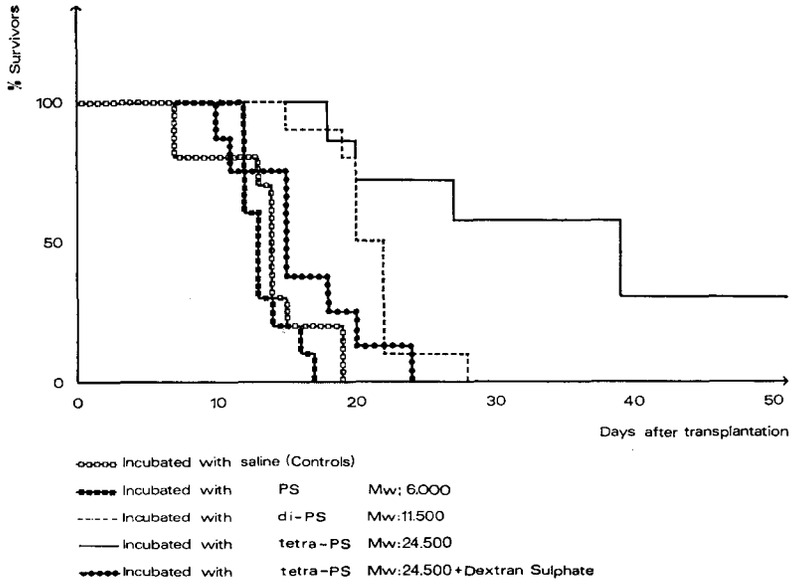


Fig. 2. Survival time of groups of 7–10 mice inoculated with tumour cells which had been incubated with protamine preparations of increasing mol wt. The effect of subsequent dextran sulphate treatment is also shown.

PS are lost during the polymerization process. Thus, the only variable molecular parameter is the mol wt.

The effect of incubating JB-1 ascites tumour cells with PS, di-PS and tetra-PS before transplantation to AK/Aa mice is seen in Fig. 2. The survival time increased with increasing mol wt of the PC used, *i.e.* monomer PS had no inhibitory effect on tumour growth; di-PS had a slight effect, while tetra-PS more than doubled the average survival time as compared with the control group. In another similar experiment, tetra-PS increased the average survival time more than fourfold, while di-PS and PS had no effect. In general, solid tumours developed in 50 % of the "long-term" survivors.

After DS treatment, the tumour cells which had been incubated with the most potent PS, tetra-PS, almost restored total malignancy as compared with the untreated controls, indicating that the cells were not killed during the *in vitro* incubation period.

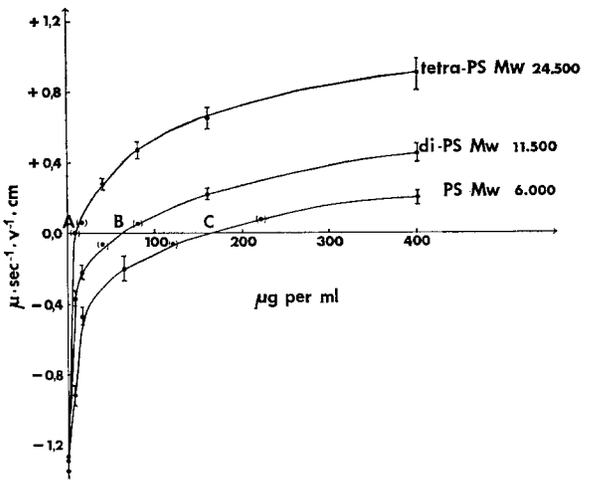


Fig. 3. Effect of protamine and its dimer and tetramer forms on the electrophoretic mobility of JB-1 ascites tumour cells. Temp 25°C. Measured in PBS–sorbitol buffer 1:4, ionic strength = 0·035, cell concentration 5×10^5/ml. SD for each measurement is shown in the figure. Mobility determinations are uncertain when approaching zero, which is indicated with brackets. The points of intersection A, B, C, are useful to illustrate the differences in relative binding affinity.

The influence of PS, di-PS and tetra-PS on the electrophoretic mobility of JB-1 tumour cells is shown in Fig. 3. It is seen that the initial negative mobility of the cells is neutralized and changed into a positive mobility, indicating a positively charged cell surface. This is obtained very easily in the presence of di-PS and tetra-PS, whereas PS has to be present in very high concentrations before a positive surface charge is obtained. Addition of equivalent amounts of DS to JB-1 cells treated with tetra-PS restored normal electrophoretic mobility of the cells.

If it is assumed that the surface-charge density is identical for cells of equal mobility then the points of intersection with the line indicating zero mobility A, B, C in Fig. 3 may be used as an expression of the relative affinity of the various protamines for the cell surface. Thus, if 1/C relates to the relative affinity of PS for the cell surface, it is seen that 1/B and 1/A indicates increasing affinity with increasing mol wt.

DISCUSSION

The present results show that polymerization of PS which exhibits no tumour toxic effect, converts this polycation into a highly tumour toxic compound. It may be suggested that the success on treatment of cancer with a formaldehyde-protamine complex (reported by O' Meara [8] probably may be due to some degree of polymerization. Polymerization based on peptide bond formation, such as that resulting from CDI mediated reactions, are clearly superior to formaldehyde crosslinking since the resulting aggregates of PS are as soluble as PS and consists of well defined polymerizates. The present results of the CDI polymerization may be explained by an end-to-end coupling of the PS molecules, leading to long flexible polymers, with a high affinity for anionic binding sites.

The electrophoretic measurements show that the binding affinity increases significantly with increasing mol wt. Similarly, the ability of PS, di-PS and tetra-PS to inhibit the growth of JB-1 ascites tumour increases with the mol wt. Thus, the effect on growth and mobility both increases with molecular size. Since the effect on mobility is a surface effect, it can be assumed that effect on growth is related to binding of the polycations to the cell surface.

Another indication for interaction of PSs and the cell at the cell-surface level is the fact that both the tumour toxic and the electrokinetic effects of tetra-PS on the cells can be reversed by DS, which, because of its great affinity for PS and PS derivatives, provokes release of the PSs from the cell surface with subsequent precipitation of the polyanion–PS aggregate.

The present results suggest that PCs interact in a mol wt dependent manner with the surface of the cells which is in accordance with the observations of Ryser [6] and with earlier experiments with DEAE-dextrans of varying mol wt [14]. By this interaction of the cell

surface with strong, positively charged polybases, conformational alterations of the cell membrane architecture is induced, leading to alterations of cellular activity which in the present study resulted in increased tumour toxic effect.

REFERENCES

1. G. Ruhenstroth-Bauer, The normal and pathological haemocytopherogram of man. In *Cell Electrophoresis*. (Edited by E. J. Ambrose), p. 66. Churchill, London (1965).
2. J. N. Mehrishi, Effect of lysine polypeptides on the surface charge of normal and cancer cells. *Europ. J. Cancer* **5,** 427 (1969).
3. A. Katchalsky, Polyelectrolytes and their biological interactions. *Biophys. J.* **4,** 9 (1964).
4. S. E. Kornguth, M. A. Stahmann and J. W. Anderson, Effect of polylysine on the cytology of Ehrlich ascites tumor cells. *Exp. Cell Res.* **24,** 484 (1961).
5. A. Katchalsky, D. Danon and A. Nevo, Interactions of basic polyelectrolytes with the red blood cell. II. Agglutination of red blood cells by polymeric bases. *Biochim. Biophys. Acta (Amst.)* **33,** 120 (1959).
6. H. J.-P. Ryser, A membrane effect of basic polymers dependent on molecular size. *Nature (Lond.)* **215,** 934 (1967).
7. V. C. Shah and P. Reilly, Effect of histones, other basic proteins and some antibiotics on the transplantability of mouse mammary tumours. *Nature (Lond.)* **213,** 403 (1967).
8. R. A. Q. O'Meara and M. J. O'Halloran, Protamine derivatives in the treatment of advanced carcinoma of the breast. *Lancet* **ii,** 613 (1963).
9. H. Fraenkel-Conrat and H. S. Olcott, Reaction of formaldehyde with proteins. II. Participation of the guanidyl group and evidence of crosslinking. *J. Amer. chem. Soc.* **68,** 34 (1946).
10. J. Bichel, A transplantable plasma cell leukemia in mice. *Acta path. microbiol. scand.* **29,** 464 (1951).
11. O. H. Lowry, Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).
12. H. Rosenberg, A. H. Ennor and J. F. Morrison, The estimation of arginine. *Biochem. J.* **63,** 153 (1956).
13. H. Tareyama, Method of colloid titration. (A new titration between polymer ions). *J. Polymer Sci.* **8,** 243 (1952).
14. E. B. Thorling, B. Larsen and H. Nielsen, Inhibitory effect of DEAE-dextran on tumour growth. 3. Effect of charge density and molecular size. *Acta path. microbiol. scand.* **79,** 81 (1971).

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Letter to the Editor

An Improved Method for Cytogenetic Studies of Solid Murine Tumors

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WHEN CONVENTIONAL methods are used, it is very difficult to obtain from solid murine tumors a sufficient number of metaphases to carry out cytogenetic studies. When colchicine is injected *in vivo* or added directly to the tumor cell suspension, very few mitoses are harvested. Moreover, the first passages of *in vitro* cultures yield few metaphases. The method described herein obviates these difficulties by culturing the tumor cells within a diffusion chamber implanted in the peritoneal cavity of syngeneic mice.

Diffusion chambers were prepared as described by Capalbo *et al.* [1] using Lucite rings and 0·22 μm Millipore filters. The tumors studied were fibrosarcomas or anaplastic sarcomas which had developed subcutaneously in BALB/c mice, at the site of implantation of a foreign body consisting of a hollow cylinder of Lucite or glass, with an average latency of 11 months [2]. The tumors were homogenized in TC199 medium and 0·2 ml of a cell suspension containing $1{\cdot}5 \times 10^6$ cells were loaded into each diffusion chamber which was then introduced into the peritoneal cavity of a mouse. Six to eight BALB/c mice of either sex, 2–3 months old, were used for each study. After 8 days, the animals were sacrificed and the diffusion chambers removed. They were placed in 0·5 % Pronase (Pronase B grade, Calbiochem) for 30 min at room temperature in order to dissolve the fibrin clot. The contents of 4 chambers were pooled and centrifuged at 800 rev/min. The pellet was resuspended in TC199 containing 1 μg/ml colchicine (Demecolcid) and incubated for 5 hr at 37°C after which it was treated with a hypotonic solution of 0·6 % sodium citrate for 15 min at 37°C. Finally, the cells were fixed twice in a 3 : 1 methanol–acetic acid solution, smeared, air-dried and stained with Giemsa. Successive passages were carried out by pooling the cell contents of the 2–4 remaining chambers, resuspending the centrifuged sediment in TC199 and loading $1{\cdot}5 \times 10^6$ viable cells into a new batch of diffusion chambers to be implanted into the peritoneal cavity of new hosts. This procedure was repeated every 8 days until the desired number of metaphases had been accumulated.

The tumor cells enclosed within the diffusion chamber remained viable and proliferated. As can be seen in Table 1, primary cultures harvested on Day 8 yielded from 10 to 30 metaphases and an equal or often greater number could be obtained in each successive passage, every 8 days. These results were never obtained when these tumors were processed directly. The tumor cells retained their morphological and cytogenetic characteristics, such as modal number and chromosomal markers throughout the successive passages.

The main advantage of this method is obviously the good yield of metaphases unobtainable by other means, in such a short period of time, in fibrous tumors. *In vitro* cultures of these tumors often fail and whenever successful, the number of metaphases necessary

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Table 1. *Metaphases obtained from tumor cells cultured within* 0·22 *μm Millipore diffusion chambers implanted in the peritoneal cavity of BALB/c mice*

	Number of metaphases					
	Primary	Passages				
Tumor	culture	1	2	3	4	Total
P193	15	20	15	25	27	102
P119	10	0	15	16		41
P143	30	40	45			115
P249	25	31	34			90

for karyotyping can only be accumulated after several passages, involving at least 2 months time, implicating the risk of cell transformation. This method can also be applied to the study of allogeneic and xenogeneic, including human, tumors, as well as to established *in vitro* cell lines; in the latter case, the number of metaphases usually increases to 70–80 in a pool of 3–4 diffusion chambers [3].

In conclusion, the *in vivo* culturing of tumor cells within Millipore diffusion chambers gives a good yield of metaphases in a relatively short length of time.

REFERENCES

1. 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).
2. C. Dosne Pasqualini, L. Sen, F. Saal, L. Schwartz and L. Z. Tkaczevski, Tumor development in mice bearing a plastic cylinder and inoculated with human neoplastic cells. *J. nat. Cancer Inst.* **51,** 283 (1973).
3. L. Rumi, S. B. Salum y A. E. Bachmann, Estudios citogenéticos en líneas celulares humanas cultivadas en cámaras de difusión en el ratón. *Med. Deporte y Trab.* **33,** 579 (1973).

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Letter to the Editor

The Effects of Tumours in Suppressing *Haemobartonella Muris* in Splenectomized Rats*

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IT IS WELL known that the majority of rats are infected with *Haemobartonella muris*. However, no overt signs of the disease in rats can be seen unless they are splenectomized. Very soon after the operation, 60–90% of splenectomized rats develop anaemia due to haemolysis. Haemoglobinuria appears to be the first clear sign of the disease which leads to death within about 10 days after the operation. The disease can be cured with arsenobenzenes.

An old and apparently forgotten finding is that tumour-bearing rats survive splenectomy in good health but later die from the tumour itself [1–3]. This led to the conclusion that the tumour either prevents the development of haemobartonellosis or promotes haematopoesis in such a way as to make it possible for splenectomized rats to compensate for the loss of red blood cells produced by haemolysis. A further well-founded explanation of the phenomenon was that anaemia in tumour-bearing rats induces the outflow from the erythropoetic tissues of immature erythroblasts which are insusceptible to *Haemobartonella muris* [3]. It may also be assumed that *Haemobartonella muris* and the effects produced by a growing tumour counteract each other in some respect. However, these views give no explanation about the role of the spleen in protecting normal infected rats from the disease and death. Therefore, it appeared reasonable to analyse the phenomenon once again.

In the experiments, male Wistar rats were studied. The tumours were sarcoma induced by subcutaneous injections of 3,4-benzpyrene (BP) [4], hepatic carcinoma induced by feeding rats with 4-dimethylaminoazobenzene (DAB) [4] and subcutaneous transplantable Yoshida ascites sarcoma (YAS). Subcutaneous BP sarcoma as well as YAS were about 1·5 cm in diameter and hepatic carcinoma was clinically palpable at the time of splenectomy. Usually about 90% of the rats fed DAB developed canaliculocellular carcinoma in the liver, the remainder had hepatocellular carcinoma.

At the time of splenectomy, tumour-bearing rats were clinically well and no death from the tumour was expected to ensue within a week or so. The observation time lasted three weeks and in the course of it, only a small part of the animals were expected to succumb to death from their tumours.

The results are presented in Table 1. As may be seen, non-tumour-bearing splenectomized rats who developed haemobartonellosis died within one week after the operation. Only one of the sick rats survived until the 14th postoperative day. Eighty-two per cent of non-tumour-bearing splenectomized rats succumb to the disease. The rats bearing the sarcoma induced by 3,4-benzpyrene, when splenectomized displayed the mortality similar to that in non-tumour-bearing rats. An increase in the overall per cent mortality within three weeks (90%) in comparison with non-tumour-

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Table 1. Mortality of tumour bearing Wistar rats after splenectomy

Tumour	No. of rats	Percentage mortality of rats after splenectomy (Days after operation) 1–7	8–14	15–21	Total percentage mortality within 3 weeks
Non-tumour-bearing rats	60	48	1		82
BP-sarcoma*	57	45	4	2	90
DAB-carcinoma†	59	12	4	5	36
YAS‡	59	12	4	7	39

*Subcutaneous sarcoma induced in rats by subcutaneous injections of 3,4-benzpyrene.
†Hepatic carcinoma (canaliculocellular or hepatocellular) induced by feeding rats with 4-dimethylaminoazobenzene.
‡Subcutaneously inoculated Yoshida ascites sarcoma.

bearing splenectomized rats can be ascribed to deaths caused by the tumour growth particularly in rats having died after the first postoperative week.

The rats with hepatic carcinoma induced by feeding with 4-dimethylaminoazobenzene, and those with subcutaneously growing Yoshida ascites sarcoma were significantly resistant to the development of haemobartonellosis after splenectomy. If it is maintained that some of these tumour bearing rats died from the growing tumour, then the counter effect of these tumours against a haemobartonellosis infection becomes stronger.

Characteristic inclusions in the red blood cells of sick rats were almost regularly found in the non-tumour- and BP-sarcoma-bearing rats that developed haemobartonellosis after splenectomy. However, in the operated animals bearing DAB-carcinoma or YAS these inclusions were seen only in some of the sick rats belonging to that group. Some of these rats also showed signs of haematuria which in most of them disappeared in a few days together with red cells inclusions. All tumourous rats succumbed to their tumours after a three week observation period.

Since the analysis of the blood smears of tumour-bearing splenectomized rats showed no immature proerythrocytes or erythroblasts, the hypothesis that the protective effect might be due to these cells cannot be supported.

However, it appears legitimate to conclude that any tumours in rats cannot take over the role of the spleen in counteracting the development of haemobartonellosis but some forms of tumour have this property.

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REFERENCES

1. F. Blumenthal and H. Auler, Mit Milzbrei von Tumorraten erzeugte Tumoren. *Z. Krebsforsch.* **24,** 285 (1926).
2. H. Hirschfeld and F. P. Tinozzi, Vergleichende Untersuchungen über die Folgen der Splenektomie bei gesunden und Tumorraten. *Z. Krebsforsch.* **26,** 304 (1928).
3. W. Kikuth, Ueber den Einfluss von Jensen-Sarkom auf die infektiöse Rattenanämie und ihre Erreger, *Zbl. Bakt. I. Abt. Orig.* **109,** 51 (1928).
4. N. Allegretti and B. Vitale, Induction of experimental allergic encephalomyelitis and tumor occurrence in rats treated with 4-dimethylaminoazobenzene and 3,4-benzpyrene, *Cancer Res.* **26,** 950 (1966).

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Effects of Intravenous BCG in Normal Dogs and in Dogs with Spontaneous Osteosarcoma*

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Abstract—*BCG was injected by the intravenous, intraperitoneal and intrathoracic routes into normal dogs and intravenously into dogs bearing spontaneous osteosarcoma which were treated by amputation or X-irradiation.*

Following intravenous injection, there was a transient pyrexia not usually exceeding 1°*C.*

The main pathological lesions produced were granulomas in the liver and lungs, and follicular lymphoid hyperplasia. In 6 *dogs with spontaneous osteosarcomas, amputation was performed and i.v. injections made of BCG or BCG plus X-irradiated autologous tumour cells. Survival times were greater than those seen in* 10 *dogs amputated for osteosarcoma but not given BCG.*

INTRODUCTION

THERE is considerable interest and research into the possible value of BCG in the immunotherapy of cancer. Intravenous BCG will protect Ha/ICR Swiss mice against the growth of transplanted sarcoma 180 tumours, will increase the survival of Swiss mice with Erlich ascites tumour and will give complete protection against carcinoma 755 in C57 hybrid mice [1].

In rats, pulmonary growths produced by i.v. injections of methyl cholanthrene-induced sarcoma cells were controlled or suppressed by specific immuno-stimulation with BCG/cell inocula at subcutaneous sites or by i.v. treatment with BCG alone. The response was dependent on the immunogenicity of the treated tumour so that, in comparison, i.v. injection of BCG enhanced, rather than suppressed pulmonary growth of transferred cells from several weakly immunogenic tumours. These included a spontaneously-arising sarcoma, an epithelioma, and a carcinogen-induced mammary carcinoma [2]. These studies also supported the concept that localisation of BCG at the tumour site is a necessary requirement for tumour suppression.

The effects of BCG on various facets of the immune response against polyoma virus in rats was also studied. Administration of BCG at the time of rat polyoma isografting, or two weeks previously, inhibited tumour growth and resulted in an increased level of cell-mediated immunity. A similar inoculation of BCG at the time when the tumour isograft had already grown out to a palpable nodule did not inhibit the tumour, but instead, caused enhanced growth [3].

Active immunotherapy with BCG and/or vaccination from a pool of allogeneic leukaemic "lymphoblasts" pretreated with formalin or irradiated *in vitro* has been given to patients with acute lymphoblastic leukaemia. Mathé, Amiel, and co-workers [4] first treated these patients by chemotherapy to reduce the number of tumour cells. Their results demonstrated the efficacy of active immunotherapy

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in the control of acute lymphoblastic leukaemia in man.

The danger that immunotherapy might actively stimulate the formation of antibodies that block existing host immunity and accelerate the neoplastic process has made for cautious advances in the treatment of human clinical cases. Complications of BCG immunotherapy in patients with cancer have been minimal following injections made intradermally. However, intratumour injections have led to pyrexia and granulomatous hepatitis [5, 6].

At the suggestion of Baldwin, [7] we injected BCG i.v. into dogs bearing spontaneous malignant bone tumours. Osteosarcoma in dog and man is equally malignant [8]. However, as there was no literature describing the effects of i.v. BCG in the dog we initially injected the vaccine into normal experimental dogs. Francis [9] found that following the i.v. injection of *virulent* bovine or human bacilli in dogs, the alveoli became filled with macrophages and other inflammatory cells and that there was hyperplasia of perivascular and peribronchial lymph nodules. The objective in clinical cases of canine osteosarcoma was to prevent or delay the onset of metastases which commonly occur within 2–5 months following amputation [8].

MATERIAL AND METHODS

1. *Bacillus Calmette Guerin (BCG)*

Freeze-dried BCG vaccine (percutaneous) was supplied by Glaxo Laboratories Ltd., Greenford, Middlesex, England. The contents of one ampoule of the vaccine, containing between 50—250 $\times 10^6$ organisms, were made up to 5 ml with normal saline and administered to dogs irrespective of size.

2. *Experimental dogs*

Ten clinically normal, adult, healthy, cross-bred dogs (15–20 kg) were injected intradermally with 0·1 ml BCG and 0·1 ml PPD, and on reading the test 3 days later, all were found to be negative.

Five dogs were injected i.v., three dogs intrathoracically and two dogs i.p. with the contents of one vial of BCG on either 3 or 4 occasions at 1 or 2 week intervals (Table 1).

Table 1. *Response to BCG and PPD in experimental dogs given BCG parenterally*

Dog. No.	Route of injection	No. of injections	Time between injections (weeks)	Response to PPD and BCG intradermally after 3 or more doses of BCG	Time between first injection and euthanasia (weeks)
1	Intravenous	4	1, 1, 2	—	8
2	Intraperitoneal	4	1, 1, 2	—	8
3	Intrathoracic	4	1, 1, 2	+ + +	8
4	Intravenous	3	2, 2	—	11
5	Intravenous	3	2, 2	—	11
6	Intravenous	3	2, 2	—	11
7	Intravenous	3	1, 1	+	35
8	Intraperitoneal	3	1, 1	+	35
9	Intrathoracic	3	1, 1	+ + +	35
10	Intrathoracic	3	1, 1	+ + +	35

Key: — negative; + weakly positive; + + + strongly positive

Following the injection, the rectal temperature was measured with an ELab Electrical Thermometer for periods up to 36 hr post injection. Blood counts were made at frequent intervals. Following three or more injections at 1 or 2 week intervals, intradermal BCG and PPD tests were made. Euthanasia was performed on groups of dogs at 8, 11 and 35 weeks following the first injection and histological examination made on many organs and tissues.

3. *Dogs bearing spontaneous osteosarcomas injected with BCG*

Thirteen dogs with spontaneous osteosarcomas of the long bones were studied. In one dog, a Retriever, there was already radiographic evidence of pulmonary metastasis when treatment was begun, but in all others chest radiographs were initially normal.

The dogs showing no radiographic evidence of metastases were divided into two groups. In 6 dogs the affected limb was amputated, and in the other 6 the tumour was irradiated, using a linear accelerator as previously described [10].

This division was based entirely upon the

wishes of the owners, some owners being unwilling to permit amputation.

Between 10^6–10^8 autologous tumour cells, X-irradiated *in vitro* with 10,000 R and mixed with the contents of one ampoule of BCG, were injected i.v. into four dogs following amputation, all other dogs receiving BCG alone. Further i.v. injections of BCG alone were made at intervals into all dogs, most injections being preceded by the intramuscular injections of an antihistaminic drug Mepyramine Maleate (Anthisan, May and Baker) (Tables 2 and 3).

4. *Dogs bearing spontaneous tumours not receiving BCG*

The only 5 dogs with spontaneous osteosarcoma in which amputation was performed at the Cambridge University Veterinary Hospital during the last 10 years and in which no other therapy was attempted have been used as control animals. These unselected dogs were 2 Labradors (femur and radius), a terrier (tibia), a mongrel (radius) and a Dalmatian (humerus). As an additional comparison, the results in 5 dogs in which the tumour-bearing limb was amputated and one

Table 2. *BCG therapy in dogs with spontaneous osteosarcoma. Primary tumour irradiated*

	Breed	Site of primary tumours	Total tumour dose X-irradiation Rad	Time interval between BCG Injections (weeks)	Survival time (weeks)	Comments
1.	St. Bernard	Distal radius	4760	1, 2, 4, 8, 8	30	Dog well. Euthanasia at request of owner. No evidence of lung metastases radiographically.
2.	Wolfhound	Distal radius	4500	1, 2, 4, 8, 8	34	Euthanasia because of local recurrence. No evidence of metastasis.
3.	Wolfhound	Distal tibia	4500	1, 2, 4	8	Euthanasia because of local recurrence. No evidence of metastasis.
4.	Labrador	Distal femur	4500	1, 2, 4, 8	14	Euthanasia because of local recurrence but extensive metastasis also present.
5.	Pyrenean	Distal radius	3500	1, 2, 4, 8	15	Dog died after showing respiratory distress. No post mortem available.
6.	Pyrenean	Distal radius	4000	1, 2, 4	16	Local recurrence. Lung metastases and hypertrophic pulmonary osteoarthropathy.

Table 3. *BCG therapy in dogs with spontaneous osteosarcoma. Leg amputated.*

	Breed	Site of primary tumour	Time interval between BCG injections (weeks)	Survival time (weeks)	Number of X-irradiated cells injected i.v.	Comments
7.	Labrador	Right distal radius	1, 1, 2	7	Nil	Lung metastases already present. Treatment had no obvious effect. Euthanasia.
8.	Boxer	Right femur	1, 2, 4, 8, 8, 8, 8	52	Nil	Died with advanced pulmonary metastases and hypertrophic pulmonary osteoarthropathy.
9.	Great Dane	Left distal femur	1, 2, 4, 8, 8, 8, 8	53+	Nil	Alive and well. No radiographic evidence of metastases.
10.	Great Dane	Left distal radius	1, 2, 4, 8, 8, 8, 8	53+	10^6	Alive with radiographic evidence of one lung metastasis.
11.	Wolfhound	Left distal tibia	1, 2, 4, 8, 8	25	10^7	Died with multiple metastases in lungs.
12.	Retriever	Left proximal tibia	1, 2, 4, 8, 8, 8, 8	46+	5×10^8	Alive and well. No radiographic evidence of metastasis.
13.	Welsh Collie	Left distal radius	1, 2, 4, 8, 8	16	10^8	Euthanasia due to multiple lung metastases.

lung received X-irradiation (2 × 600 R) have been used [10].

The results in 6 dogs where the primary tumour was X-irradiated and i.v. BCG given have been compared with the results in 16 dogs where the primary tumour was similarly irradiated and one lung received X-irradiation (2 × 600 R) [10].

RESULTS

1. *Toxic effects*

No experimental dog showed an anaphylactic-like response following i.v. injections of BCG, but this occurred in one dog with osteosarcoma injected with BCG for the second time. Subsequently, osteosarcoma cases were injected with mepyramine maleate 20 min before the BCG injections and none showed an anaphylactic-like response. In 2 dogs, part of the BCG injection was perivascular and resulted in a granuloma which healed in 6–8 weeks.

Rectal temperature rises of 0·5–1°C a few hours following injection were common, but in only one dog (No. 10, Gt. Dane) was pyrexia greater than this (1·7°C). Owners reported clinical dogs to be dull with decreased appetite the day following the injection but, except in one case where a severe pharyngitis lasting 10 days developed (No. 9, Gt. Dane), animals soon returned to normal. In no less than four clinical cases was a continued, greatly increased, appetite noted. In a Wolfhound (case 2), which was a Veterinary Surgeon's own dog and where accurate recording was possible, the food intake, compared with that of 3 months before the onset of the tumour, doubled without appreciable increase in body weight.

2. *Intradermal BCG and PPD tests, and haematological studies*

In 5 normal experimental dogs which had each received 3 or 4 i.v. injections of BCG, one dog became weakly positive. All three dogs receiving intrathoracic injections became strongly positive. One of 2 dogs injected i.p. became weakly positive (Table 1). In five clinical dogs tested after receiving three or more i.v. injections of BCG, three became weakly positive.

There were no significant changes in the total or differential wbc counts.

3. *Pathological examination*

Apart from changes directly associated with a recurrent primary, or multiple secondary tumours (the latter usually being found in the lungs but in one animal also being present in the kidneys), there was little of significance to be seen on gross *post mortem* examination in those dogs where BCG had been injected i.v. In the experimental dogs given intrathoracic injections, numerous white lesions 2–3 mm in diameter were visible on the pleura. In dogs injected i.p. no gross changes were seen in the peritoneum.

Histological changes related to BCG were essentially similar in both the experimental dogs and dogs with spontaneous osteosarcoma.

In the lungs, the most obvious feature was a marked perivascular mono nuclear reaction, the predominant cell types being macrophages and lymphocytes, with a few plasma cells also present. In addition, small granulomas, with a central zone of 15–20 macrophages, surrounded by a thin rim of lymphocytes, were seen throughout the lung parenchyma. In general, these were not so numerous in the clinical as in the experimental dogs. In those animals which had pulmonary metastases, there was no indication of an inflammatory reaction around tumour nodules.

Lesions in the liver were very marked and consisted of an infiltration of the portal connective tissues by mononuclear cells, again with macrophages predominating. Small, focal granulomas were numerous in this organ in all cases examined. An interesting feature in the liver was the presence of masses of haemosiderin in both the periportal and focal macrophages, indicating the presence of previous haemorrhage into the organ. This was not seen in the lung lesions.

The only other feature of note was the presence of marked follicular lymphoid hyperplasia in the tonsils and bronchial lymph nodes. Special staining techniques failed to reveal intact tubercle bacilli in any organ examined but in some of the granulomas in the liver, small particles of acid fast material, which may have represented disintegrating organisms, were seen.

4. *Development of lung metastases*

Tables 2 and 3 show the results of BCG therapy alone or combined with X-irradiated autologous tumour cells. No effect on the continued growth of tumour in the lungs was seen in the one dog where metastases were radiographically visible at the start of therapy.

Survival times in the 6 dogs which had the primary tumour irradiated ranged from 6–34 weeks (average 19 weeks). Two showed no

evidence of metastases on post-mortem examination (including the one surviving 34 weeks) and one had no radiographic evidence of metastasis. Previous results [9] in 16 dogs where the primary tumour was similarly X-irradiated together with a smaller dose of irradiation to one lung, showed survival times ranging from 9–52 weeks (average 20 weeks) with 5 dogs showing no metastases at the time of death.

In the 6 dogs which had no evidence of pulmonary tumours at the time of amputation, the average survival times is in excess of 41 weeks. Three died or were killed as a result of multiple lung metastases at periods ranging from 16–52 weeks after amputation. Three dogs are still alive, one having radiographic evidence of one metastasis after 53 weeks and 2 with no evidence of metastases 46 and 53 weeks after amputation.

Survival times in 5 dogs receiving amputation alone ranged from 6–17 weeks (average 11 weeks).

Survival times in 5 dogs where amputation was performed plus X-irradiation of one lung ranged from 13–49 weeks (average 23 weeks) [9].

DISCUSSION

Although the repeated i.v. injections of BCG may be considered a hazardous procedure, all clinical dogs now dead were destroyed as a direct result of tumour growth and in no case were they showing clinical signs attributable to the effects of BCG at the time of death. Immediate post injection anaphylaxis apparently can be completely prevented by the prior administration of antihistamine.

It is somewhat surprising that in the experimental dogs receiving BCG by various routes, only 5 exhibited a reaction to PPD and that in 2 of these animals the reaction was weak. More work is planned on the response to PPD and the immunological status of treated dogs.

All of the lung and liver granulomas induced by the BCG may eventually heal with minimum damage to the dog. Indeed, in some dogs injected, the granulomas were relatively infrequent.

Dogs treated with BCG and in which the primary tumour was X-irradiated and left *in situ* had a survival time approximately equal to dogs in which the primary was irradiated together with one lung. Unfortunately, in these two groups of dogs, euthanasia was usually performed because of pain caused by recurrence of the primary tumour with the natural history of the condition incomplete. However, there was no indication that enhancement occurred in dogs treated with BCG. Survival time was not decreased and metastasis did not become more prevalent.

In those cases where the limb was amputated, there appears to be an increased survival time in the dogs treated with BCG or BCG+X-irradiated autologous tumour cells. Four dogs of 6 lived approximately one year after amputation. The final survival time may be much greater as 3 dogs are still alive, 2 being clinically and radiographically normal. These survival times are much better than those of the 10 dogs amputated for osteosarcoma but not given BCG (Range 6–49 weeks, average 18 weeks). American figures for survival following amputation alone showed that 17/29 dogs were dead in < 14 weeks, a further 6 in 17–32 weeks and with 6/29 surviving more than 48 weeks [11].

The number of animals treated with BCG is small but the initial results indicate no enhancement and a possible delay in the appearance of metastases. They are encouraging enough for us to continue with a larger series of animals, including dogs, in which mastectomy has been performed for malignant mammary tumours. Enthusiasm for therapy of mammary tumours will be tempered by observations indicating that in some models, BCG increases rather than decreases the rate of growth of mammary tumours [12]. If further results do confirm that metastases are delayed following their infiltration by BCG with or without the addition of autologous X-irradiated cells there would be an increased interest in this type of therapy in human malignant tumours perhaps using X-irradiated BCG or fractions of the bacterial cell.

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REFERENCES

1. L. J. Old, D. A. Clark and B. Benacerraf, Effect of Bacillus Calmette-Guerin infection on transplanted tumours in the mouse. *Nature (Lond.)* **184,** 291 (1959).
2. R. W. Baldwin and M. V. Pimm, BCG Immunotherapy of pulmonary growths from intravenously transferred rat tumour cells. *Brit. J. Cancer* **27,** 48 (1973).
3. S. C. Bansal and H. O. Sjogren, Effects of BCG on various facets of the immune response against polyoma tumours in rats. *Int. J. Cancer* **11,** 162 (1973).
4. G. Mathe, J. L. Amiel, L. Schwarzenberg, M. Schneider, A. Cattan, J. R. Schlumberger, M. Hayat, F. de Vassal, Active immunotherapy for acute lymphoblastic leukaemia. *Lancet* **1,** 697 (1969).
5. F. C. Sparks, M. G. Silverstein, J. S. Hunt, C. M. Haskell, Y. H. Pilch, D. L. Morton, Complications, of BCG immunotherapy in patients with cancer. *New Engl. J. Med.* **289,** 827 (1973).
6. J. S. Hunt, M. G. Silverstien, F. C. Sparks, C. M. Haskell, Y. H. Pilch, D. L. Morton, Granulomatous hepatitis: a complication of BCG immunotherapy. *Lancet* **II,** 820 (1973).
7. R. W. Baldwin, Personal communication (1973).
8. L. N. Owen, *Bone Tumours in Man and Animals.* p. 29. Butterworths, London (1969).
9. J. Francis, *Tuberculosis in Man and Animals.* p. 211. Cassel London (1958).
10. L. N. Owen and D. E. Bostock, Prophylactic X-irradiation of the lung in canine tumours with particular reference to osteosarcoma. *Europ. J. Cancer* **9,** 747 (1973).
11. R. S. Brodey, Surgical treatment of canine osteosarcoma. *J. Amer. vet. med. Ass.* **147,** 729 (1965).
12. W. F. Piessons, F. L. Lachapelle, N. Legros and J. C. Heuson, Facilitation of rat mammary tumour growth by BCG. *Nature (Lond.)* **228,** 1210 (1970).

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Separation of the Specific Antigen of a Mouse Lymphoma from Histocompatibility Antigens

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Abstract—*A rabbit antiserum against the mouse lymphoma EL4 was rendered EL4-specific by exhaustive absorption with syngeneic (C57BL/6) spleen cells. This absorbed serum retained some (complement mediated) cytotoxicity for EL4 cells; its ability to protect syngeneic mice from tumour challenge is described elsewhere. This serum was used to monitor the extraction and fractionation of the specific antigen of EL4 cells which is absent from the host mouse. EL4 cell membranes were solubilized autolytically and by papain digestion and fractionated by gel-filtration and ion-exchange chromatography. By both methods, the EL4 tumour specific antigen could be separated from H-2 histocompatibility antigens, in gel filtration by its greater size and in ion-exchange by its higher charge.*

INTRODUCTION

IT IS BECOMING increasingly clear that a host's immunological status in relation to a tumour is a crucial matter in prognosis although the full significance of the different parameters is far from clear in patients and in autochthonous tumours in animals. The nearest model which has sufficient versatility for immunochemical studies is the syngeneic animal tumour. The relationship of a tumour-specific antigen to features of malignant growth is not yet understood, but in any case it is a particularly important feature in that it is one which can be taken advantage of for specific therapy.

In this paper we describe some properties of a molecule carrying tumour specificity of a carcinogen induced lymphoma EL4 of C57BL/6 mice [1]. This specificity has already been located in the tumour cell membranes [2]. The antigen has now been separated from mouse H-2 histocompatibility antigens, properties of which have been described [3], and has features distinguishing it from other gene products of the major histocompatibility complex [4, 5]. The importance of defining an extraction procedure and the properties of such a substance is of two kinds, theoretical and practical.

From the theoretical point of view it is necessary to know, when a mouse model is used for asking clinically relevant questions, that the putative tumour specificity is not a minor histocompatibility difference between mouse and tumour, arising in the course of transplantation. EL4 lymphoma is claimed to have as many as three features distinguishing it immunologically from its host mouse, "X", [1] "E" [6] and "L" [7]. It is not clear whether one or all of these is carried by the substance described below, but this molecule is relevant because it has been identified throughout by an antibody in a serum which protects C57BL/6 mice against EL4 lymphoma; the EL4 specific cytotoxic antibody titre of this serum correlates with its protective effect [8]. Therefore, we are not impressed with the thesis of histocompatibility deviation.

From the practical point of view, the use of a tumour specific antigen as immunogen for

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induction of tumour specific antibodies is important because there are logistic problems in absorbing out unwanted (non-tumour-specific) antibodies when whole cells are used. For the mouse, rabbit antisera require 3000–5000 spleens/100 ml serum for adequate absorption [9] and goat anti-human melanoma sera, require about 7 kg of human spleen/litre (O'Neill, to be published). "Adequate" absorption necessarily means complete absorption when passively administered antibody is used to provide specificity *e.g.*, for a combined use of drug and antibody (DRAB effect), [10, 11]. The substance we describe now has not yet been tested as an immunogen for raising "protective" antibody because yields are small and essentially "analytical" quantities have been obtained so far. Immunogenicity will be reported upon in a later communication. A comprehensive review of the immunology of chemically induced tumours has recently appeared.[12].

MATERIAL AND METHODS

Animals and tumour

EL4 is an ascites lymphoma which was induced by dimethylbenzanthracene in C57-BL mice [1]. We maintained it by serial i.p. passage in C57BL/6 mice; the mice used were bred in our own colony. Rabbits were bought from accredited dealers. EL4 is X+, E+, L+, as already indicated, but TL-ve [13–15] hence non-thymic in origin. The lethal dose for C57BL/6 mice is about 5 cells, given by i.p. or s.c. routes [8].

Immunizations

Xenoantisera were made in rabbits, which received 3 injections i.v. at 10 day intervals each of approximately 10^8 cells and bleedings were taken 10 days after the 3rd injection. The procedure was adopted on the basis of experiments showing a peak of reactivity for three parameters in the 3rd bleeding of a series of 5 tested independently; each bleeding was made just before the next immunizing injection [8]. These parameters were: (a) protection of syngeneic mice by passive immunotherapy; (b) maximal cytotoxicity for EL4 cells (after suitable absorption); and (c) maximal immunofluoresence of EL4 cells.

Absorption

Antisera were made EL4 specific by repeated absorptions with normal syngeneic spleen cells (up to 6 times at 10 spleens/ml were sometimes necessary) as previously described in detail [8]. Absorption was taken to completion, such that no complement mediated cytotoxicity remained for normal C57BL/6 lymph node cells or thymocytes, even under the most exacting test conditions, i.e. rabbit (not guinea pig) complement, neuraminidase pretreatment of target cells and an incubation period extended from 1 to 3 hr. These absorbed sera reacted, however, with EL4 target cells as shown in Fig. 1.

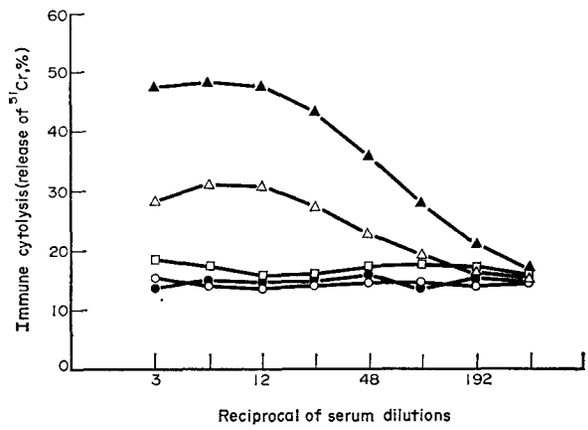


Fig. 1. Cytotoxic reactions of tumour-specific antisera: complement mediated reactions and 3 hr incubation time, measured by release of ^{51}Cr from labelled cells. Anti-EL4 rabbit serum absorbed with normal H-2^b spleen cells tested against H-2^b (C57BL/6) lymph node cells as target, ○——○*; with EL4 cells as target,* △——△*; with H-2^b lymph node cells treated with neuraminidase as target,* □——□*; with EL4 cells treated with neuraminidase as target,* ▲——▲*. H-2 antiserum from H-2^b mice immunized with H-2^d cells, tested against neuraminidase treated EL4 cells,* ●——●.

Cytotoxicity tests

These were carried out as previously described [9] using guinea pig serum as routine complement source (rabbit sera when necessary but they are usually too toxic as a complement source for EL4 cells). Cell death was quantitated by release of ^{51}Cr from labelled target cells. Inhibition tests to measure antigenic activity were carried out by diluting putative antigen (H-2 or tumour specific) in antiserum at a 75% lytic dilution in excess complement. Column fractions were generally scanned by single point assays in a similar system, fractions being concentrated when necessary if diluted too far by the column eluting buffers.

Fractionation of antigen

This was done by standard methods already described [3]; particular conditions of solubilization, gel filtration and ion-exchange chromatography are described below.

RESULTS

A group of 120 C57BL/6 mice was injected i.p. with 10^5 EL4 cells and the ascitic fluid containing the lymphoma cells washed out 12 days later into a volume of 800 ml saline/citrate (batch "EL4/4"). This was diluted to 1800 ml with distilled water to give 0·4% NaCl and left overnight at 4° to elute the plasma membrane fraction [16]. Cells were removed by centrifuging at 1500 rev/min for 10 min and the supernatant was centrifuged at 30,000 × *g* for 1 hr to sediment the crude membrane material ("eluate"), which was resuspended in water at about 20 mg (dry weight) per ml. The cells were eluted twice more in a similar way, but at 0·3% (NaCl) and the 3 eluates were combined to give 1·22 g of material suspended in 76 ml of water.

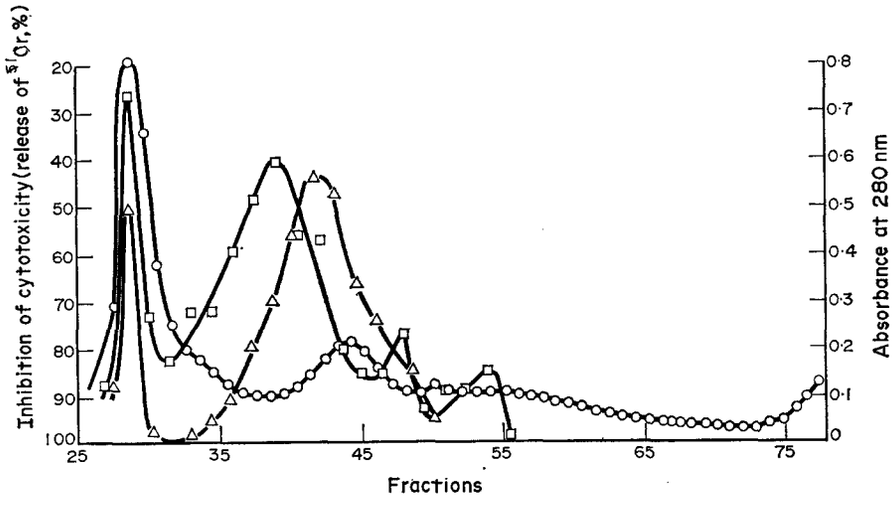


Fig. 2. Sephadex G200 gel filtration of solubilized EL4 cell membrane preparation. Optical density at 280nm, ○——○; location of H-2 antigen, monitored by H-2^a anti-H-2^b serum, measured by release of ^{51}Cr and plotted on an arbitrary scale, △——△. Location of EL4 lymphoma tumour specific antigen, monitored by rabbit anti-EL4 serum (R129/130) (absorbed to completion with normal C57BL/6 spleen cells). target cells, ^{51}Cr labelled neuraminidase treated EL4 cells, □——□.

The crude eluate was solubilized by leaving for a week at 4° and then treating with ultrasonics for 30 sec (M.S.E. 100 W ultrasonic disintegrator, amplitude 8 μ, 20 kc/sec). The solution was then centrifuged at 145,000 × *g* for 2 hr and the supernatant fraction put aside ("A"). The sediment was resuspended in water at approx. 30 mg/ml and adjusted with *M* Tris–HCl buffer, pH 8·0, to a final [0·05*M*]. To this was added freshly made cysteine HCl (0·5% w/v in 0·05 *M* pH 8 buffer) at 1% v/v, and papain (twice recrystallized, at 20 mg protein/ml) at 1% v/v, i.e. 1 :150 w/w protein. This solution was incubated at 37° for 1 hr, cooled and proteolysis stopped by the addition of 5 ml *M* iodoacetic acid adjusted to pH 8·0 with Tris–HCl buffer. This was centrifuged at 145,000 × *g* for 2 hr and the supernatant fraction ("P") put aside. The sediment was stored for other uses and the autolytic (A) and papain solubilized (P) fractions pooled, passed through a Millipore filter (0·45 μ), dialysed against water and freeze dried to give fraction AP, of 247 mg.

A sample of fraction AP, (80 mg) was dissolved in 7 ml of 0·01 *M* Tris buffer pH 7·3 and passed through a G75 Sephadex column (50 cm × 3 cm); the 10 ml fractions were scanned at 280 nm, and the excluded fractions pooled. Samples of this material were tested by inhibition of cytotoxicity in a system detecting *H*-2^b antigens (specificities 2.33.56) and in a system detecting EL4 tumour specific antigen. There was inhibitory activity in both tests, but none in a specificity control (*H*-2^b anti-*H*-2^d serum with *H*-2^d target lymphocyt es), showing that both H-2 histocompatibility antigens and tumour specificity were in the G75 excluded material. Papain was removed among the G75 retarded substances.

The G75 excluded fraction was dialysed, concentrated under reduced pressure at 4°, freeze dried and made up to 7 ml in 0·01 *M* Tris buffer pH 7·3. This was passed through a Sephadex G200 column (1 m × 3 cm) into 10 ml fractions which were scanned for H-2 and EL4 cell specific antigens by single point cytotoxic inhibition assays. The result of this experiment is shown in Fig. 2; the two antigens

are clearly distinguishable but not completely separated. However, a pool made from tubes 34 to 40 when re-run on G200 Sephadex showed a substantial "EL4 peak" and a relatively very small "H-2 peak". From this run, pools were made of: (a) excluded material; (b) the EL4 antigen reactive region; and (c) included material and after dialysis and freeze drying 32·5, 20 and 1·1 mg respectively were recovered; pool 2 retained only traces of H-2 activity.

Also from the EL4/4 AP soluble preparation, a sample of 150 mg was fractionated on Sephadex DEAE-A50 using a column 60 cm × 2 cm and eluting with a gradient from 0·05 *M* Tris buffer pH 8 to 0·05 *M* Tris pH 9 with 0·45 *M* NaCl. The 100 fractions were collected of 10 ml each and the column was then cleared with 100 ml of 1 *M* NaCl (pool 5). From the 280 nm OD pattern 4 pools were made, dialysed, freeze dried and reconstituted for test. The products, and the results of inhibition tests measuring H-2 (H-2^b, 2.33.56) histocompatibility specificity and EL4 tumour specific activity (rabbit anti-EL4 serum absorbed to give no cytotoxic reaction with normal *H*-2^b lymphocytes over a 3 hr incubation period), with H-2^b lymph node cells and EL4 cells respectively as targets, are shown in Table 1. These results could not be quantified numerically but the separation of the two activities is very clear.

Table 1. *Products of DEAE ion exchange separation of material solubilized from EL4 lymphoma plasma membranes*

Fraction	Molarity range	Wt. (mg)	Serological Specificity H2	EL4
1	0·05—0·15	9·3	nil	nil
2	0·15—0·28	29·2	+ + +	(±)
3	0·28—0·37	24·7	+	+ +
4	0·37—0·50	15·6	(±)	+ + +
5	0·50—1·00	6·2	(±)	+

Another preparation from EL4 cells (EL4/5), where the product from 140 mice was eluted in 3 vols. of 2.7 l, but otherwise prepared as for EL4/4, was solubilized autolytically and by papain digestion. These two products were not pooled. The papain product was passed through G75 Sephadex and subsequently G200 Sephadex columns and from the latter 4 pools were made: (1) excluded (24 mg); (2) retarded (mainly EL4 activity); (3) retarded (mainly H-2 activity, 6·8 mg); and (4) included (4 mg). Pool 2 was re-run and cut to separate H-2 and EL4 areas, giving 7 mg of the fraction low in H-2 activity. This was applied to a Sephadex DEAE-A50 column 70 cm × 9 mm which was eluted with 200 ml (2 ml fractions) using a gradient from 0·05 *M* Tris pH 8 to 0·05 *M* Tris pH 8 and 0·45 *M* NaCl pH 8. The 100 fractions were recovered individually by dialysis and freeze drying and concentrated by reconstitution each in 200 μl of medium suitable for cytotoxic inhibition tests. EL4 activity appeared in the 0·3 to 0·4 *M* elution region of the column.

DISCUSSION

In preceding papers [8–11] we have used mouse lymphomas as models for immunotherapy and for studies of the synergistic effect of drugs and antibodies (Drab effect). The sera originally used were alloantisera absorbed *in vivo*, but subsequently xenoantisera absorbed *in vitro* have been used. In both cases, a residual cytotoxic titre remained for tumour cell targets after total removal, by absorption with normal spleen cells, of antibodies reactive with normal syngeneic mouse lymphoid cells. This tumour directed cytotoxicity is weak and technically difficult to use, with variables not yet understood; it is nevertheless a property of sera that protect mice against tumour growth. A homologous immunogen is therefore presumed to exist that is an exclusive feature of the lymphoma cells and distinguishes them, in particular, from the host's normal lymphoid cells. Transplanted tumours in animals are under suspicion for being too far removed from clinical reality to be useful, but for the syngeneic lymphoma used in the present study (EL4), the cytotoxic titre (after absorption) varies directly with both immunofluorescence of the tumour cells (none on normal cells) and with protective capacity *in vivo* in series of bleedings of rabbits immunized with EL4 cells. From the rule of parsimony this correlation leads us to the working hypothesis that the corresponding immunogen is not only a tumour-specific antigen, but a "protective antigen" i.e. the substance under immune surveillance. This surveillance breaks down, however, to allow syngeneic tumour growth under the conditions of our test systems *in vivo* (a challenge of 10,000 lethal doses [11]). In a previous paper [2] membrane fractions of EL4 cells were shown by alloimmunization to raise "protective" antibodies, showing this antigen to be surface membrane located.

Properties of this antigen are important to study for many reasons. From the theoretical point of view we should like to know if the antigen is related to or derived from some other (e.g. histocompatibility) antigen or is an entirely different substance. These alternatives would imply (a) a defect in an existing synthetic pathway or (b) a product of a gene derepression associated with neoplasia, respectively. From the practical point of view, a purified immunogen might produce antisera with different features from those used here, e.g., they might not need such extensive absorption to render them tumour specific and might have higher titres or better binding capacity.

Lymphoma cells require a more hypotonic medium to elute the plasma membrane fraction than normal lymphoid cells [16] but otherwise, through a series of preliminary extractions, they are found to be amenable to the methods we use for preparation of transplantation antigen glycoproteins as now described, i.e. gentle proteolysis [3]. These methods have also been used previously for tumour-specific antigen extractions [14, 15, 17].

The data in this paper show that EL4 antigen differs from H-2 antigen; it is separable from H-2 by its larger size, as seen on gel filtration. We have not recognised hitherto any surface antigen eluting in this position. TL (thymus leukaemia) antigen, which can be regarded as tumour-specific, e.g. in ASLI (H-2^a) and ERLD (H-2^b) lymphomas, coincide with H-2 histocompatibility antigens on gel filtration. Other products of the major histocompatibility complex (MHC) in mice have so far proved to be approximately the same molecular size or smaller, but never larger, than the H-2 antigens used as marker on the column illustrated in Fig. 2 and the EL4 antigen is more highly charged than any so far recognised as seen from its DEAE elution point. TL antigen is itself more highly charged than H-2 [14, 15] but less so than 'R' antigen [4] and *a fortiori* less than EL4 antigen as described here.

Insufficient material is yet available to study any *in vivo* properties but the synergistic behaviour of drugs and antibodies (Drab effect) has, in our hands, occurred using antisera which are related, according to our hypothesis, to the kind of antigen described here. It is not yet clear if cross reacting antigens will take part in a Drab effect. EL4 antigen does not cross react with the lymphomas ERLD (H-2^b) or SB1 (H-2^d) [8], but EL4 did cross react with the extinct lymphoma CL2 [2]. If an antigen cross reactive between different tumours will serve for a Drab effect it could be very important clinically. Cross reactivity is not a feature of carcinogen induced tumours but is a feature of e.g. leukaemias and melanomas.

Features of EL4 tumour specific antigen distinguish it from all of the many antigens we can recognize on H-2^b lymphoid cells and supports the idea that it is a "new" substance, not a modified form of one pre-existing on normal cells. This fact validates the tumour-specific immunological basis and clinical relevance of our drug–antibody (Drab) protection tests [8, 11].

REFERENCES

1. P. A. Gorer and D. B. Amos, Passive immunity in mice against C57BL Leukosis E.L.4 by means of iso-immune serum, *Cancer Res.* **16,** 338 (1956).
2. D. A. L. Davies, Occurrence of 'X' antigenic specificity in histocompatibility antigens prepared from leukaemic cells. *Brit. J. exp. Path.* **44,** 546 (1963).
3. M. Hess and D. A. L. Davies, Basic structure of mouse histocompatibility antigens. *Europ. J. Biochem.* **41,** 1 (1974).
4. D. A. L. Davies and M. Hess, A new alloantigen genetically linked to the major histocompatibility locus of the mouse. Isolation and partial characterization. *Nature (Lond.)* **250,** No. 5463 (1974).
5. N. A. Staines, K. Guy and D. A. L. Davies, Passive enhancement of mouse skin allografts: specificity of the antiserum for major histocompatibility complex antigens. *Transplantation* **18,** No. 2 (1974).
6. T. Aoki, B. Stück, L. J. Old, U. Hammerling and E. Deharven, E. Antigen: a cell surface antigen of C57BL Leukaemias. *Cancer Res.* **30,** 244 (1970).
7. J. C. Leclerc, J. P. Levy, B. Varet, S. Oppenheim and A. Senik, Antigenic analysis of L strain cells: a new murine leukaemia-associated Antigen, "L". *Cancer Res.* **30,** 2073 (1970).
8. D. A. L. Davies, A. J. Manstone and S. Buckham, Protection of mice against syngeneic lymphomas: 1, use of antibodies. *Brit. J. Cancer* **30,** 297 (1974).

9. D. A. L. Davies and G. J. O'Neil, *In vivo* and *in vitro* effects of tumour specific antibodies with chlorambucil. *Brit. J. Cancer* **28,** 285 (1973).
10. D. A. L. Davies, S. Buckham and A. J. Manstone, Protection of mice against syngeneic lymphomas: 2, collaboration between drugs and antibodies. *Brit. J. Cancer* **30,** 305 (1974).
11. D. A. L. Davies, The combined effect of drugs and tumor-specific antibodies (DRAB) in protection against a mouse lymphoma. *Cancer Res.* **34,** 3040 (1974).
12. R. Baldwin, Immunological aspects of chemical carcinogenesis. *Adv. Cancer Res.* **18,** 1 (1973).
13. E. A. Boyse, E. Stockert and L. J. Old, Isoantigens of the *H*-2 and *TLa* loci of the Mouse. *J. exp. Med.* **128,** 85 (1968).
14. D. A. L. Davies, E. A. Boyse, L. J. Old and E. Stockert, Mouse isoantigens: separation of soluble TL (thymus-leukemia) antigen from soluble H-2 histocompatibility antigen by column chromatography. *J. exp. Med.* **125,** 549 (1967).
15. D. A. L. Davies, B. J. Alkins, E. A. Boyse, L. J. Old and E. Stockert, Soluble TL and H-2 antigens prepared from a TL positive leukaemia of a TL negative mouse strain. *Immunology* **16,** 669 (1969).
16. D. A. L. Davies, Mouse histocompatibility isoantigens derived fromnormal and from tumour cells. *Immunology* **11,** 115 (1966).
17. R. W. Baldwin and D. Glaves, Solubilization of tumour-specific antigen from plasma membrane of an aminoazo-dye-induced rat hepatoma. *Clin. exp. Immunol.* **11,** 51 (1972).

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Cure of a Mouse Lymphoma with Radio-iodinated Antibody*

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Abstract—*A cell-surface localizing heterologous antibody against the mouse EL4 lymphoma could be bound to varying amounts of ^{131}I without interfering with the reactivity of the antibody with EL4 cells. Injection of the radio-iodinated immunoglobulin into C57BL mice pre-inoculated with EL4 lymphoma cells led to partial or complete tumour suppression depending upon the amount of radio-iodinated antibody injected. Injection of comparable amounts of the immunoglobulin alone or of ^{131}I bound to normal globulin did not cause any tumour inhibition. The anti-tumour antibody did not have any radio-sensitizing effect on the EL4 cells.*

INTRODUCTION

WE HAVE demonstrated before that even if not therapeutically effective by themselves, those antibodies which localize on the surface of tumour cells might be successfully used as "homing" carriers for radiotherapeutic agents [1] or cytotoxic drugs [2, 3]. We have reported that a heterologous antibody against the mouse Ehrlich Ascites Carcinoma (EAC) could carry enough ^{131}I to EAC cells to prevent the growth *in vivo* of tumour cells after exposure to the radio-iodinated antibody *in vitro* [1]. We report here varying degrees of tumour inhibition and even complete tumour suppression by injections of radio-iodinated antibody in mice pre-inoculated with EL4 lymphoma cells. These results also show that the degree of tumour inhibition is related to the amount of ^{131}I carried by the antibody. Injection of comparable amounts of antibody alone or of comparable amounts of ^{131}I bound to normal rabbit globulin did not cause any tumour inhibition. Contrary to our observation in the EAC model [4], exposure of EL4 cells to the heterologous anti-EL4 antibody did not render the tumour cells more radio-sensitive.

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MATERIAL AND METHODS

Tumour

EL4 lymphoma which originated in 1945 in a C57BL/G0 mouse treated by Gorer with dimethyl benzanthracene was maintained from 1946 onwards at the Chester Beatty Research Institute, London [5] from where we obtained the tumour in 1969 and maintained it by serial intra-peritoneal passage in C57BL/6J mice purchased from the Jackson Memorial Laboratories (Bar Harbor, Maine).

Anti-tumour sera

For the production of anti-EL4 serum, freshly obtained, viable (i.e. at least 98% trypan blue impermeable) tumour cells were washed three times with phosphate buffered saline (PBS, 0·1 M, pH 7·1) at 4°C. Each member of groups of 6 female New Zealand white rabbits, weighing 2·5–3 kg each, were intramuscularly injected into both flanks and both shoulders with a total of 4×10^8 EL4 cells suspended in 4 ml of PBS and mixed with an

equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). The injections were repeated after one week. From one week after the second set of injections, the rabbits were injected intramuscularly twice a week with 10^8 viable EL4 cells per rabbit without any adjuvant. Three days after the tenth injection of 10^8 tumour cells alone, 5 ml of blood were obtained from the ear vein of each rabbit (test bleeding). The resulting sera were separately absorbed with homogenates of normal C57BL mouse tissues and tested thereafter, by immunofluorescence for the presence of specific anti EL4 antibody as described below. Rabbits showing titers of 1/64 or above of specific anti EL4 antibody in their test bleeding serum were exsanguinated. The resulting sera were pooled, inactivated at 56°C for 30 min and then successively absorbed with washed homogenates of liver, lungs, kidney and spleen from normal adult C57BL/6 mice. Absorption with every organ homogenate (homogenate to serum ratio 1 : 3 by volume) was carried out at 37°C for 4 h followed by another 8 h absorption at 4°C. Absorbed sera were separated from the homogenates by centrifugation (20,000 × *g*) for 2 h at 4°C. Absorptions were repeated (usually 2 absorptions each with liver, lungs, and kidney homogenates and 3 to 4 absorptions with spleen homogenate) till the serum reacted as tested by immunofluorescence, only with EL4 cells and neither with cryostat sections of normal C57BL mouse tissues nor with suspensions or smears of C57BL/6 lymphoid cells derived from lymph nodes, spleen and thymus, B/6 melanoma cells maintained in C57BL/6 mice, 2 lines of AKR/J lymphoma cells (which originated spontaneously in about one year old AKR/J female mice in our laboratory and are maintained by serial passage in AKR/J mice) and Ehrlich ascites tumour cells maintained in BALB/C mice. Immunofluorescence staining was done by "sandwich" method using fluorescein isothiocyanate labelled goat anti-rabbit globulin with appropriate controls [1–3]. When tested *in vitro* by the dye-exclusion method [1], this anti-tumour globulin had no cytotoxic effect on EL4 cells in the presence of normal rabbit or guinea pig serum as sources of complement.

Preparation and administration of radio-iodinated anti-EL4 and normal rabbit serum globulins

The anti-EL4 serum (after being rendered tumour specific by absorptions with homogenates of normal C57BL mouse tissues) and normal rabbit sera were fractionated with 33·3% saturated ammonium sulphate. The resulting globulin fractions, after being freed of ammonium sulphate with repeated dialysis against PBS, were conjugated with varying amounts of ^{131}I using chloramine T. Usually 4·4 ml of PBS containing 10 mg/ml of anti-EL4 or normal rabbit globulins were mixed at 4°C with varying amounts of carrier and reductant free ^{131}I dissolved in 0·1 M NaOH (NEZ 035A, New England Nuclear, Boston, Mass.). To this mixture 1·1 mg of chloramine T dissolved in 0·2 ml of PBS was added (i.e. globulin to chloramine T ratio 40:1). The mixture was shaken at 4°C for 30 min after which the reaction was stopped by adding 1·1 mg of sodium metabisulfite in 0·2 ml PBS. Unbound ^{131}I was eliminated by repeated dialysis (at least 6 times in 24 h) against PBS, till virtually no ^{131}I activity diffused out in the PBS. About 40 to 55% of ^{131}I bind to rabbit globulins by this procedure. ^{131}I activity was counted in a well-type gamma counter (Picker Medicals, Arlington, Va.). Auto-radiography was performed on formalin fixed smears of EL4 cells using Eastman Kodak NTB3 emulsion. Binding of all radio-iodine labelled antibody to EL4 cells was confirmed by immunofluorescence and auto-radiography [1, 3]. To block the uptake of any dissociated ^{131}I by thyroid tissue all mice were given 1% Lugol's iodine in drinking water at least 5 days prior to the administration of ^{131}I.

Testing for radio-sensitization

For testing whether the anti-EL4 globulin had any radio-sensitizing effect on EL4 cells, aliquots containing 10^6 freshly collected EL4 cells were suspended either in 0·5 ml of 0·01 M phosphate buffered saline (PBS, pH 7·1), or in 0·5 ml PBS containing either 4 g of immune rabbit globulin (IRG), or 4 mg of normal rabbit globulin (NRG) and then exposed to 0, 400, 600, 800, 1000, 1250, 1500, 1750 and 2000 rads of γ-rays from a ^{137}Cs γ-beam (Atomic Energy of Canada Ltd.) at a dose rate of 4·1 rad per second [4, 6]. After 0·5 h incubation, the cells were washed and groups of 5 mice were injected i.p. with the lymphoma cells that had been incubated in PBS, in PBS + NRG or PBS + IRG. Viability of tumour cells was routinely determined using trypan blue exclusion test before their inoculation into mice.

RESULTS

The results of i.p. injection of radio-iodinated anti-EL4 antibody on the survival of C57BL

mice pre-inoculated with 10^4 EL4 cells are presented in Table 1. It is worth pointing out here that i.p. inoculation of 10^4 cells in C57BL mice invariably leads to the development of ascites tumour and subsequent death of the tumour inoculated mice. Intraperitoneal inoculation of as few as about 10 EL4 cells leads to ascites formation and death of the tumour inoculated mice, the period of survival of the tumour inoculated mice, however, depends upon the number of tumour cells inoculated, i.e. with higher number of inoculated cells, the tumour appears earlier and the survival time is reduced accordingly (Table 2).

Five hundred and forty-four μCi of ^{131}I bound to 4 mg of IRG injected 2 h after tumour inoculation (Group 2) and 499 μCi of ^{131}I bound to 4 mg of IRG injected 24 h after tumour inoculation (Group 6) completely suppressed tumour growth in 4 out of 5 mice and prolonged the survival of the remaining mouse in each group. 592 μCi of ^{131}I bound to 4 mg of NRG (Group 3), 4 mg of NRG alone (Group 5), 4 mg of IRG alone (Group 4) injected 2 h after tumour inoculation or 4 mg of IRG alone injected 24 h after tumour inoculation (Group 7) did not prolong the survival of the EL4 inoculated mice. On the other hand; injection of 612 μCi of ^{131}I bound to 6 mg of IRG injected 48 h after tumour inoculation (Group 8) or 1020 μCi of ^{131}I bound to 8 mg of IRG injected 72 h after

Table 1. *Survival of groups of C57BL mice injected with different doses of radio-iodinated antibody or radio-iodinated normal globulin after i.p. inoculation of syngeneic EL4 lymphoma cells. Each group contained five adult female mice*

Group No.	Treatment* ^{131}I μCi/globulin (mg)†	Interval (h) between tumour inoculation and beginning of treatment	Survival (No. of days ± s.d.)
1	0/0 P.B.S.	2	17·2 ± 0·7
2	544/4 I.R.G.	2	†1 died 143 days after tumour inoculation ‡4 survived 1 yr
3	592/4 N.R.G.	2	19·8 ± 1·4
4	0/4 I.R.G.	2	17·4 ± 1·8
5	0/4 N.R.G.	2	16·6 ± 0·8
6	499/4 I.R.G.	24	§1 died 36 days after tumour inoculation ‡4 survived more than 1 yr
7	0/4 I.R.G.	24	18·0 ± 1·6
8	612/6 I.R.G.	48	**All survived more than 200 days
9	0/6 I.R.G.	48	18·2 ± 0·4
10	715/4 N.R.G.	48	22·2 ± 1·9
11	1,020/8 I.R.G.	72	**All survived more than 200 days
12	489/4 I.R.G.	72	‡1 survived more than 1 year, 1 died 20 days, 2 died 22 days, and 1 died 29 days after tumour inoculation
13	0/8 I.R.G.	72	19·2 ± 1·6

*Treatment consisted of one i.p. injection of 1 ml phosphate buffered saline containing the stated amounts of ^{131}I activity and globulin.

†Due to advanced putrefactive changes, the cause of death in this mouse could not be established. This mouse had an enlarged spleen at autopsy and, therefore, probably died of EL4 lymphoma.

‡These mice died of natural causes (senility). Histological examination of internal organs did not reveal any tumour.

§The internal organs of this mouse were widely infiltrated with EL4 lymphoma cells.

**Four of the 5 surviving mice from these groups were challenged with i.p. inoculation of 10^4 EL4 cells, 230days after the first tumour inoculation.

Table 2. Survival of groups of C57BL inoculated i.p. with varying numbers of EL4 lymphoma cells. Each group contained at least ten adult female mice

Number of EL4 inoculated	Survival (days ± s.d.)
10	26·6 ± 6·84
10^2	23·4 ± 2·60
10^3	20·0 ± 1·0
10^4	18·4 ± 0·55
10^5	15·6 ± 1·81
10^6	12·8 ± 0·83
10^7	12·4 ± 0·89
5×10^7	11·0 ± 0·00

tumour inoculation (Group 11) led to complete tumour suppression. However, tumour inhibition was less effective when the amount of radio-iodinated antibody injected 72 h after tumour inoculation was lowered to 489 μCi of ^{131}I bound to 4 mg of IRG (Group 12). Similarly, 612 μCi of ^{131}I bound to IRG injected 48 h after tumour inoculation (Group 8) completely protected 5 out of 5 mice, whereas, 544 or 499 μCi of ^{131}I bound to IRG injected even earlier, i.e. 2 or 24 h after tumour inoculation (Groups 2 and 6 respectively) led to complete tumour suppression in only 4 out of 5 mice in each group. These results suggest that the degree of tumour suppression is related directly to the amount of ^{131}I carried by IRG. Control groups consisting of mice injected 48 hr after tumour inoculation with 715 μCi of ^{131}I bound to 4 mg of NRG (Group 10), or 6 mg of IRG alone (Group 9) or injected 72 h after tumour inoculation with 8 mg of IRG alone (Group 13) did not show any significant tumour inhibition.

The peripheral blood of mice injected with radio-iodinated IRG or NRG, examined on the 7th and 19th days after tumour inoculation, revealed lymphopenia. On autopsy, the mice which were injected with radio-iodinated NRG and died of disseminated EL4 lymphoma showed atrophy of spleen and lymphoid tissue, though almost all internal organs were heavily infiltrated with lymphoma cells. The EL4 inoculated mice which were treated with radio-iodinated IRG and survived for more than a year did not reveal the presence of EL4 lymphoma when their internal organs were histologically examined. However, the spleens of these mice were much smaller than those in a control group of mice of the same age.

Four mice from each group 8 and 11 were challenged with an i.p. inoculum of 10^4 EL4 cells 230 days after first tumour inoculation. The period of survival of these mice (18·8 ± 1·6 days) was not different from the survival of a control group of previously untreated C57BL mice of comparable age inoculated i.p. with 10^4 EL4 cells (19·2 ± 0·6 days), thus showing that these surviving mice did not have at this time adequate immunity to protect them against 10^4 EL4 lymphoma cells.

Exposure of EL4 lymphoma cells to the anti-tumour globulin did not appear to have any effect on the radio-sensitivity of the lymphoma cells (Table 3).

Table 3. Survival of groups of C57BL mice inoculated i.p. with EL4 cells suspended in either PBS or PBS+ IRG and exposed to varying doses of γ-radiation. Each group contained five to ten adult C57BL mice. No inoculation of EL4 cells exposed to 1750 rads or more produced tumour

EL4 cells exposed to (rads)	Mean survival time (days ± s.d.) of groups of mice inoculated with 10^6 EL4 cells suspended in PBS	PBS + IRG
0	13·8 ± 1·33	14·2 ± 1·26
400	14·4 ± 1·51	15·8 ± 1·30
800	18·6 ± 1·26	18·4 ± 0·07
1000	20·3 ± 1·71	20·8 ± 1·04
1250	24·0 ± 1·65	23·6 ± 2·88
1500	6 died, mean survival 25·1 ± 3·3 days, 4 surviving.	4 out of 5 died 24, 29, 37 and 53 days after tumour inoculation, 1 surviving.
1750	All surviving	All surviving

DISCUSSION

Three distinct tumour associated antigens have been described in EL4 lymphoma cells [7], however, the precise nature of the antigen reacting with the heterologous anti-EL4 globulin remains to be determined.

Though the EL4 lymphoma originated in a subline of C57BL mice maintained by Gorer [5], in our laboratory inoculation of as few as about 10 EL4 cells led to progressive ascites tumour formation, extensive tumour infiltration of organs and tissues and subsequent death of all C57BL/6J and another subline of C57BL mice (C57BL/Icrf subline) obtained from the laboratories of the Imperial Cancer Research Fund. There was also no difference in the survival between groups of C57BL/6J and C57BL Icrf mice after inoculations of 10^2, 10^3, 10^4 and 10^5 EL4 cells. It is relevant to note that Gorer maintained for his work a subline of C57BL mice in his own laboratory from 1934 to 1940 when he transferred some of his

breeding stock of C57BL mice to the laboratories of the Imperial Cancer Research Fund [8]. The C57BL mice which Gorer used subsequently were the descendants of the breeding stock maintained in the laboratories of the Imperial Cancer Research Fund [8]. Subcutaneous or i.p. inoculation of 10^6 EL4 cells did not produce in our laboratory any persistent or progressively growing tumour in A/J, AKR/J, BALB/CJ, C3H/HeJ or SWR/J mice. These observations show that the EL4 tumour line maintained by us has retained its strain specificity as orginally observed by Gorer [5] and also that there is no detectable histoincompatibility between the EL4 tumour line maintained by us and the experimental C57BL/6J mice as adjudged by the rate of growth and lethality of the tumour in C57BL/6J and C57BL/Icrf mice. The rabbit anti EL4 globulin used in these experiments is tumour specific only in the sense that it reacted with an antigen present on the surface of EL4 cells, but not detectable in the normal tissues of adult C57BL/6 mice or in several other mouse tumour lines examined by us. Whatever may be the precise nature of this antigen, the results of these experiments demonstrate that a cell surface localizing, non-cytotoxic antibody (similar anti-tumour antibodies are found in the serum of many cancer patients and tumour-bearing animals [9]) after radio-iodination was an effective tumour inhibitor, whereas, the antibody alone, or comparable amounts of ^{131}I bound to normal rabbit globulin had no tumour inhibitory effect. Though a cell surface localizing anti-EAC antibody acted as a potent radio-sensitizer [4], thus explaining, at least in part, the inhibition of EAC by the radio-iodinated anti-EAC globulin [1], anti-EL4 globulin did not appear to have any such radio-sensitizing effect. Tumour-free survival of C57BL mice which received injections of ^{131}I antibody 72 h after tumour cell inoculation is especially interesting, because tumour cells circulate in the blood of these mice as early as 72 h after i.p. tumour cell inoculation. This could be demonstrated by the development of EL4 ascites tumour in C57BL mice injected i.p. with blood obtained from C57BL mice which were inoculated i.p. with tumour cells 72 h before. Histological evidence of tumour invasions of different organs could be seen in these mice from 7 to 9 days after i.p. inoculation of 10^4 lymphoma cells.

Following the observation of Pressman and Keighley [10] that radio-iodinated anti-tissue antibody, after i.v. injection did actually localize in target tissues, localization of radio-iodinated anti-tumour antibodies in tumour tissue in amounts higher than in normal tissues has now been demonstrated in several mouse tumour models [11–13] and in human gliomas [14]. Our own preliminary investigations have also demonstrated similar preferential localization of radio-iodinated anti-tumour antibodies both in human and mouse solid tumours, and this might be potentially useful in cancer diagnosis by external scintillation scanning as well as in the treatment of solid tumours. The suppression of i.p. inoculated and freely circulating EL4 lymphoma cells after the injection of radio-iodinated anti-tumour antibody is more relevant to the treatment of human leukemias where cancer cells are readily accessible to the radio-iodinated antibody than to solid tumour where vascularity of tumours and the movement of radio-iodinated globulins across the vascular wall further complicate the situation [15]. Atrophy of lymphoid tissue, as observed in the mice treated with radio-iodinated IRG is usually associated with immunosuppression and susceptibility to infection. This might thus constitute, as with other forms of radiation and chemotherapy of cancer, a limitation to the possible use of radio-iodinated tumour antibodies in the treatment of cancer. Unwanted "homing" of the radio-iodinated anti EL4 globulin especially to organs rich in reticuloendothelial cells like the spleen and lymph nodes is not surprising because the anti EL4 globulin used in these experiments contained only about 1% specific anti EL4 antibody molecules. To be able to carry therapeutic or diagnostic amounts of radioactivity exclusively to tumour tissue, the specific EL4 cell localizing antibodies would have to be separated from those irrelevant globulin molecules which constitute the bulk of the anti EL4 serum globulin used in these experiments. It is hoped that radio-iodination of purer anti EL4 antibody preparations obtained by elution from EL4 cells used for absorbing out the specific anti EL4 antibody from crude anti EL4 globulin preparations like the one used in these experiments might eliminate or substantially reduce the unwanted homing of radioactivity to non-neoplastic tissue. However, it is encouraging that none of the EL4 inoculated mice which had long term tumour-free survival after treatment with radio-iodinated IRG either died or showed any other deleterious effect of damage to the haemopoetic or immunologic systems.

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REFERENCES

1. T. Ghose, M. Cerini, M. Carter and R. C. Nairn, Immunoradioactive agent against cancer. *Brit. med. J.* **1,** 90 (1967).
2. T. Ghose and S. P. Nigam, Antibody as carriers of chlorambucil. *Cancer* **29,** 1398 (1972).
3. T. Ghose, S. T. Norvell, A. Guclu, D. Cameron, A. Bodurtha and A. S. Macdonald, Immunochemotherapy of cancer with chlorambucil-carrying antibody. *Brit. med. J.* **3,** 495 (1972).
4. T. Ghose and M. Cerini, Radiosensitization of Ehrlich ascites tumor cells by a specific antibody. *Nature (Lond.)* **222,** 993 (1969).
5. P. A. Gorer, Studies in antibody response of mice to tumour inoculation. *Brit. J. Cancer* **2,** 372 (1950).
6. O. P. Kamra, T. Ghose and M. Mammen, Radiosensitization of EL4 lymphoma cells by a commonly used food additive—butylated hydroxytoluene (BHT). *Mutat. Res.* **21,** 293 (1973).
7. D. A. L. Davies and G. J. O'Neil, *In vivo* and *in vitro* effects of tumour specific antibodies with chlorambucil. *Brit. J. Cancer (Suppl.* 1) **28,** 285 (1973).
8. P. A. Gorer and Z. B. Mikulska, The antibody response to tumour inoculation. Improved methods of antibody detection. *Cancer Res.* **14,** 651 (1954).
9. R. T. Smith, Possibilities and problems of immunologic intervention in cancer. *New Engl. J. Med.* **287,** 439 (1972).
10. D. Pressman and G. Keighley, The zone of activity of antibodies as determined by the use of radioactive tracers; the zone of activity of nephritoxic anti-kidney serum. *J. Immunol.* **59,** 141 (1948).
11. A. E. Reif, Studies on the localization of radiolabelled antibodies to a mouse myeloma protein. *Cancer* **27,** 1433 (1971).
12. M. J. Izzo, D. J. Buchsbaum and W. F. Bale, Localization of an ^{125}I labelled rat transplantation antibody in tumours carrying the corresponding antigen. *Proc. Soc. exp. Biol. (N.Y.)* **139,** 1185 (1972).
13. J. A. Kellen and J. S. Lo, Localization of ^{125}I labelled antibodies against tumour associated proteins from experimental rat mammary neoplasms. *Res. Commns. chem. Path Pharmac.* **5,** 411 (1973).
14. E. D. Day, Myelin as a lucus for radioantibody absorption *in vivo* in brain tumours. *Cancer Res.* **28,** 1335 (1968).
15. E. D. Day, Immunological distribution analysis. In *Research in Immunochemistry and Immunobiology* (edited by J. B. G. Kwapinski), Vol. 3, p. 41. University Park Press, Baltimore (1973).

Europ. J. Cancer Vol. 10, pp. 793–800. Pergamon Press 1974. Printed in Great Britain

Metabolic Controls in Precancerous Liver—VI. Studies *In Vivo* and *In Vitro* on Feedback Regulation of Δ-aminolevulinic Acid Synthetase in Ethionine-fed Rats*

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Abstract—*We have studied induction of δ-aminolevulinic acid (ALA) synthetase by the drug allylisopropylacetamide and the ability of exogenous heme to repress such induction in normal rat liver, in the livers of rats fed the hepatocarcinogen, ethionine and in host livers and hepatomas of rats bearing Morris hepatomas* 5123*C*, 7794*A and* 9618*A. We also measured changes in tyrosine aminotransferase.*

In experiments in vivo, *ALA synthetase was shown to be inducible in normal liver, in ethionine-treated liver, in host livers of hepatoma-bearing rats and in hepatoma* 5123*C. Under our conditions it was non-inducible in hepatomas* 9618*A and* 7794*A. In normal and host livers injected heme repressed drug-stimulated induction, but in ethionine-treated livers and in hepatoma* 5123*C this feedback-type repression by heme was absent. This apparent defect in metabolic control in both cancerous and precancerous liver parallels the similar loss of feedback control of cholesterol synthesis previously reported.*

In contrast to findings in vivo *for ethionine-fed rats, heme repression was found to be fully operative when studied* in vitro, *i.e. in liver cells isolated from ethionine-treated rats. The possible significance of this difference between findings* in vivo *and* in vitro, *in relation to the nature of ethionine-induced changes in precancerous liver, is discussed.*

INTRODUCTION

IT IS GENERALLY recognised that in animal cells as well as in microorganisms, feedback mechanisms have an important role in regulating key synthetic reactions. This has led to the suggestion by several authors, notably Potter [1], that breakdown in feedback regulation of the synthesis of important intracellular components could be of fundamental significance in carcinogenesis. The one apparently clear-cut example of such loss of feedback control in cancerous tissue is that governing cholesterol synthesis in liver tumours; the absence of normal dietary feedback control of cholesterol synthesis is a characteristic of every hepatoma yet examined [2, 3]. A similar loss of control occurs in the livers of rats treated with four different chemical hepatocarcinogens —aflatoxin, N-2-fluorenylacetamide, 3′ methyl-4-dimethylaminoazobenzene and ethionine [3]. Moreover, this loss takes place within a few weeks of beginning carcinogen treatment, i.e. many weeks or months before histologically-discernible tumours appear [3].

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Since it is important to know whether other feedback mechanisms might be similarly affected in cancerous and pre-cancerous tissue, we have now examined feedback control of the enzyme δ-aminolevulinic acid (ALA) synthetase in normal liver, in tumours and host livers of rats with transplanted Morris hepatomas, and in the precancerous livers of ethionine-fed rats.

ALA synthetase is the first and rate-limiting enzyme of the heme biosynthetic pathway, and in normal mammalian liver heme and porphyrin synthesis are controlled through changes in the level of this enzyme [4, 5]. Basal enzyme activity is low in normal liver but is rapidly induced by a wide variety of compounds [6], including the drug allylisopropylacetamide (AIA) which in rat liver causes several-fold induction over 6 hours [4, 7]. Induction of ALA synthetase by drugs is repressed if the end product, i.e. heme,* is administered at the same time as the inducing compound [8, 9]. The mechanism for control of ALA synthetase might be regarded as a characteristic differentiated function of adult liver since in other mammalian tissues, with the possible exception of kidney, AIA does not induce [10, 11]. In foetal liver the enzyme level is higher than in the adult [12, 13] and non-inducible [12, 14].

MATERIAL AND METHODS

Animals and tumours

Adult male rats, either Buffalo or Hooded Wistar, were obtained from this University's Central Animal House and maintained at constant temperature under a controlled lighting schedule. They were offered standard laboratory rations (Whiting and Chambers, Adelaide) and water *ad libitum.*

The Morris hepatomas (5123C, 7794A and 9618A) were originally imported from the United States and have been maintained in Buffalo strain rats in Adelaide for several years by serial, intra-muscular transplantation. Tumour-bearing rats were used for experiments after transplanted hepatomas had grown to about 1–2 cm diameter.

For the carcinogen studies male Wistar rats, aged 2–3 months, were fed either powdered standard ration (controls) or this mixed with 0·25 % D-L-ethionine, as previously described [15, 16].

*'Heme' is used here as a general term for iron protoporphyrin compounds. The compound used experimentally was hemin, i.e., ferric protoporphyrin.

Enzyme regulation studies in vivo

Prior to the measurement of enzyme activity, all animals were fasted for 24 or 48 hr, since induction of ALA synthetase by drugs such as AIA is largely prevented in fully-fed or glucose-fed animals [17, 18].

Groups of animals, previously treated as indicated, were used as follows. At zero time and again after 90 min they were injected i.p. with hemin (15 mg/kg) or the corresponding volume of saline. At 90 min they were also injected s.c. with AIA (300 mg/kg), or the corresponding volume of saline. After a further 6 hr all rats were killed. Livers, and where applicable transplanted hepatomas, were excised and placed immediately in ice-cold "PEST" buffer (5 mM Tris–HCl pH 7·4, 0·25 M sucrose, 0·1 mM EDTA, 0·1 mM pyridoxal-5-phosphate).

The tissue was weighed and homogenized in 9 volumes of PEST buffer using a glass/teflon Potter–Elvejhem homogenizer. Homogenate samples were frozen rapidly, using a dry ice–ethanol bath, and stored at −15°C until assayed—usually on the next day.

Enzyme studies with isolated liver cells

Cell suspensions were prepared from the livers of rats fed ethionine for 2, 3 or 4 weeks, then starved for 24 hr before use. The method of cell isolation, based on that of Berry and Friend [19], has been described in detail previously [20] and yields from each liver about 4 ml of packed cells, with almost 95 % viable cells as judged by exclusion of trypan blue. Freshly prepared cells were incubated in Waymouth medium plus 10 % foetal calf serum, and test compounds added to suspensions at zero time in a small volume of saline. After 5 hr incubation at 37°C cells were collected by centrifugation, homogenized and frozen and thawed twice before enzyme assay.

ALA synthetase

Frozen and thawed homogenates were assayed in duplicate by the colorimetric procedure described by Whiting and Elliott [21]. Briefly, thawed homogenates were incubated with glycine and pyridoxal phosphate in the presence of an exogenous succinyl CoA-generating system (succinyl CoA synthetase from *E. coli*) and 10 mM EDTA (to inhibit ALA dehydratase). ALA formed in 20 min incubations at 37°C was estimated by conversion to a pyrrole and reaction with Ehrlich reagent, with an appropriate correction for aminoacetone con-

tamination [22]. All activities were corrected for blanks in which trichloroacetic acid was added at zero incubation time. It should be noted that activities measured represent the sum of ALA synthetase activities in mitochondrial and cytosol fractions.

Tyrosine aminotransferase (TAT)

Liver homogenates, prepared as described above, were diluted 6-fold with "PEST" buffer and assayed for TAT activity by the method of Rosen *et al.* [23].

Protein

Homogenate samples were assayed for protein by the Biuret procedure [24].

Feedback control of cholesterol synthesis

Hepatic cholesterol synthesis, in the presence and absence of dietary cholesterol, was measured in control and ethionine-fed rats by the method of Horton *et al.* [16, 25], using the drug AY-9944 (*trans*-1,4-*bis*(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride). AY-9944 blocks the conversion of 7-dehydrocholesterol to cholesterol, and the level of 7-dehydrocholesterol in the blood 24 hr after an i.p. injection of AY-7794 is indicative of hepatic sterol synthesis during that time. In a normal animal this level, as would be expected, is much lower when the animal is fed a high-cholesterol diet. This reduction is a measure of the effectiveness of the cholesterol feedback system operating in that animal. The assay was used on animals, fed ethionine, comparable to those used in the study of ALA synthetase.

Materials

AIA, a gift from Hoffman-LaRoche, Basel, Switzerland, was injected as a saline solution (20 mg/ml). Hemin (Sigma, Bovine Type I) was dissolved in a small volume of 0·1 N NaOH, diluted with saline and carefully adjusted to pH 7·8 by drop-wise addition of 0·1 N HCl, the final concentration was 2 mg/ml. Other fine chemicals were purchased from Sigma. AY-9944 was a gift from Dr. Morris Givner, of Ayerst Research Laboratories, Montreal.

RESULTS

Hepatomas

The basal level of ALA synthetase in livers and tumours of normal and tumour-bearing animals *in vivo*, and changes in this level in response to an inducing drug, AIA, or inducer plus feedback repressor, heme, are shown in Table 1. The dose of AIA employed (300 mg/kg) is sufficient to give maximal or slightly less than maximal induction of ALA synthetase in normal Sprague–Dawley or Wistar rat liver [18, 26]. The double administration of hemin (15 mg/kg) used here to test for heme repression is similar to the procedure used by Marver [8] to cause almost complete repression of induction by drugs in rat liver.

Table 1. *Regulation* in vivo *of ALA synthetase in Buffalo-strain rats, with and without Morris hepatomas*

Hepatoma present	Tissue assayed		ALA synthetase (pmoles ALA/mg protein/20 min) Saline	AIA	AIA and heme
None	Liver		138 ± 40 (3)*	509 ± 90 (4)	199 ± 27 (3)*
5123C	Host liver	(Exp. 1)	154 ± 4 (3)†	261 ± 23 (4)	133 ± 31 (3)*
		(Exp. 2)	150 ± 9 (2)*	435 ± 78 (3)	161 ± 23 (3)*
	Hepatoma	(Exp. 1)	126 ± 15 (3)†	220 ± 16 (4)	264 ± 21 (3)‡
		(Exp. 2)	301 ± 6 (2)	449 ± 56 (3)	436 ± 48 (3)‡
7794A§	Host liver		232 (2)	875 (2)	240 (2)
	Hepatoma		277 (2)	320 (2)	290 (2)
9618A§	Host liver		80 (2)	371 (2)	279 (2)
	Hepatoma		179 (2)	179 (2)	158 (2)

Fasted rats (48 hr) were injected with saline, AIA, or AIA plus heme, as indicated. After 6 hr, homogenates of hepatomas and/or livers were prepared, frozen and thawed and assayed for ALA synthetase. Full experimental details are given in Material and Methods. Values listed are mean activities ± s.e.m. with the number of experimental animals given in parentheses. Hepatomas are listed in order of decreasing rate of growth and increasing degree of differentiation.

*$P < 0.05$
†$P < 0.01$
‡N.S.
} statistical significance of difference from "AIA" value.

§24 hr fast only.

In the livers of normal Buffalo rats, AIA administration resulted (Table 1) in about 3-fold induction over 6 hr: this induction was almost completely repressed by heme. From our studies with tumour-bearing animals (Table 1), three principle observations arise concerning ALA synthetase regulation. First, the low ALA synthetase level characteristic of normal adult liver and most other tissues except bone marrow [6] was also found in tumours and host livers of rats bearing the three hepatomas studied. Secondly, enzyme inducibility in tumours and host livers varied with the tumour involved. Under our conditions, ALA synthetase was non-inducible in hepatomas 7794A and 9618A, both relatively slow growing, well differentiated hepatomas with normal or near-normal karyotypes [27]. These tumours resemble foetal liver, in that ALA synthetase was non-inducible [12, 14], but the higher-than-adult levels of enzyme reported in foetal liver [12, 13] were not found. In the hypertetraploid, more rapidly growing hepatoma 5123C [27], ALA synthetase was induced approximately 1·5–2-fold over 6 hr by AIA. Although small, this induction was significant, suggesting that non-inducibility of ALA synthetase is not an invariable property of hepatomas [28, 29] nor is it related to the extent of overall deviation from normal liver. Inducibility in host livers was reduced in rats bearing hepatoma 5123C. Our preliminary studies with hepatomas 7794A and 9618A, however, in contrast to the general findings of Bonkowsky *et al.* [28, 29], suggest that the presence of these tumours had little effect on inducibility in host livers.

The third and probably most significant finding was that in hepatoma 5123C, induction of ALA synthetase was not repressed by heme, although heme caused 100% repression in the host livers of the same rats. The ability of exogenous heme to repress drug-stimulated induction could not be gauged for hepatomas 7794A and 9618A, where under our conditions AIA failed to induce.

Ethionine-fed rats

Feedback control of cholesterol synthesis was tested at 0, 2 and 4 weeks of ethionine feeding (Table 2). In confirmation of previous results [16], this control was significantly lost from the livers of the carcinogen-treated animals. For rats subjected to an identical ethionine-feeding schedule, our findings on the regulation *in vivo* of ALA synthetase are shown in Table 3 and of TAT in Table 4. Although inducibility of ALA synthetase was virtually

Table 2. Defective feedback control of cholesterol synthesis in ethionine-fed rats

Treatment	No. of animals	Cholesterol synthesis (per cent feedback control) Period of treatment (weeks) 0	2	4
None	7	34 ± 6		19 ± 2
Ethionine-fed	9	45 ± 7		162 ± 14†
Ethionine-fed	6		67 ± 5*	144 ± 24‡

In each animal cholesterol synthesis was measured, as described in Material and Methods, when the animal was on a low cholesterol diet and again after 2–3 days of feeding a high (5%) cholesterol diet. Feedback control is expressed as the rate of synthesis with the high cholesterol diet as a percentage of the rate with the low cholesterol diet, i.e. a low value indicates effective feedback control. The values given are mean percentages ± S.E.M. Three separate groups of rats were each tested twice.

*$P < 0.05$
†$P < 0.001$
‡$P < 0.01$
} statistical significance of difference from control value.

Table 3. Regulation in vivo *of ALA synthetase in livers of Wistar rats after various periods of ethionine feeding*

Ethionine (0·25%)	ALA synthetase (pmoles ALA/mg protein/20 min) Saline	AIA	AIA and heme
None	280 ± 16 (5)*	1128 ± 69 (5)	426 ± 41 (6)*
2 weeks	203 ± 5 (4)†	750 ± 95 (4)	694 ± 27 (4)‡
4 weeks	382 ± 37 (5)*	1169 ± 78 (5)	1007 ± 58 (5)‡

Experimental procedure was as in Table 1, except that normal Wistar rats were used after varying periods of ethionine-feeding. Values given are means ± S.E.M. for the numbers of animals shown in parentheses.

*$P < 0.001$
†$P < 0.01$
‡N.S.
} statistical significance of difference from "AIA" value.

Table 4. Regulation of tyrosine aminotransferase in livers of Wistar rats after various periods of ethionine feeding

Ethionine (0·25%)	TAT (nmoles PPA*/mg protein/10 min) Saline	AIA	AIA and heme
None	105 ± 21 (5)†	522 ± 67 (5)	820 ± 41 (6)‡
2 weeks	244 ± 29 (4)§	572 ± 110 (4)	894 ± 59 (4)§
4 weeks	176 ± 58 (5)‡	613 ± 87 (5)	1080 ± 76 (5)‡

The same homogenates as used in Table 3 were assayed for TAT, as described in Material and Methods. Values are means ± S.E.M. for the numbers of animals given in parentheses.

*PPA—*p*-hydroxyphenylpyruvic acid.

†$P < 0.001$
‡$P < 0.01$
§$P < 0.05$
} statistical significance of difference from "AIA" value.

Table 5. Regulation of ALA synthetase in liver cells isolated from ethionine-fed rats

Additions to incubation medium	ALA synthetase (pmoles ALA/mg protein/20 min)				
	(Weeks of feeding ethionine: 0·25%)				
	2	3		4	
	I	II	III	IV	Av.
Zero time		304	428	188	307 ± 69 (3)*
None	409	291	373	419	385 ± 20 (7)*
Hemin (2×10^{-5}M)		272	322		305 ± 31 (3)*
AIA ($1{\cdot}2 \times 10^{-3}$M)	991	775	692	920	854 ± 50 (7)
AIA + hemin (7×10^{-6}M)	470			465	467 ± 23 (3)*
AIA + hemin (1×10^{-5}M)	376			370	372 ± 37 (4)*
AIA + hemin (2×10^{-5}M)	321	155	354	299	301 ± 34 (7)*

In four separate experiments (I–IV) isolated liver cells from rats fed ethionine were incubated for 5 hr at 37° in Waymouth medium, plus 10% fetal calf serum and dibutyryl cyclic AMP (5×10^{-5}M), and then assayed for ALA synthetase. Figures given are means from (generally) duplicate incubations. The average values are means ± S.E.M., for the numbers of incubations in parentheses.

*$P < 0{\cdot}001$ Statistical significance of difference from "AIA" value.

unaffected by carcinogen treatment, its feedback repression by heme was lost, even by 2 weeks. The studies on TAT showed that although the basal TAT level was higher in ethionine-treated rats, the absolute increases in activity due to administration of AIA or AIA plus heme were similar in control and carcinogen-fed animals.

In order to examine further this apparent loss of feedback control over ALA synthetase, we proceeded to measure enzyme induction and repression in isolated cells. Our findings on enzyme regulation *in vitro* (Table 5) presented a substantially different picture from that seen above *in vivo*. The results obtained with intact liver cells isolated from animals on the carcinogenic diet did not differ from those obtained previously [20] with cells isolated from animals on the control diet, i.e. in both cases AIA induced the enzyme ALA synthetase and heme repressed this induction.

DISCUSSION

Previous studies *in vivo* on feedback regulation of hepatic cholesterol biosynthesis by dietary (i.e. exogenous) cholesterol have left little doubt that this control, while present in normal liver, is absent from every one of a large variety of hepatomas tested, as well as from livers of hepatocarcinogen-treated animals [2, 3]. If, as has been postulated [1–3], this defect provides an indicator (direct or indirect) for some fundamental step in the carninogenic process, then it is important to look for other, similar defects in metabolic control.

There is now good evidence [30] that the inhibitory effect of dietary cholesterol involves first, inhibition of the activity, and second, repression of the synthesis of the major rate-controlling enzyme of the cholesterol biosynthetic pathway, namely β-hydroxy-β-methylglutaryl coenzyme A reductase. The inhibition of hepatic heme synthesis by exogenous heme provides a similar example of feedback control on the level of a key rate-controlling enzyme, in this case the first enzyme in heme and porphyrin biosynthesis, ALA synthetase [8, 9]. Our present studies *in vivo* suggest that as with feedback regulation by cholesterol, feedback repression by heme may be absent in cancerous and precancerous liver; heme failed to exert its normal repression of drug-stimulated induction of ALA synthetase in both the livers of rats fed the hepatocarcinogen, ethionine and in the one Morris hepatoma (5123C) where the enzyme was found to be inducible. Since in the other hepatomas tested ALA synthetase activity was low and non-inducible, statements in such cases on whether "heme repression" is operative or not could only be based on speculation about the relationship between heme repression and other factors which might determine inducibility of ALA synthetase.

If the apparent loss of hepatic feedback control observed *in vivo* were to be considered an indication of early events in chemical carcinogenesis it was clearly important to determine whether the loss reflected changes in intracellular controls of gene expression or changes at some other level. We therefore examined the effects of added heme on AIA-

stimulated induction in isolated liver cells prepared from the livers of ethionine-fed rats. While in such rats *in vivo* injected heme failed to repress, addition of heme to cells, isolated and incubated in suspension, resulted in repression almost identical to that in control cells from untreated rats.

Apparent loss of feedback control *in vivo* but not *in vitro*, observed here for ALA synthetase, may also apply to feedback control of cholesterogenesis. For instance, hepatoma 7288C fails to exhibit dietary feedback by cholesterol when tested *in vivo* [31], but in cells isolated from this hepatoma and maintained in tissue culture, i.e. HTC cells, added lipid, including cholesterol, does depress cholesterogenesis [32, 33]. It will thus be of particular interest to investigate cholesterol feedback in cells isolated from livers of ethionine-treated rats.

So far we have relatively little information as to why, in ethionine-treated rats, heme repression of ALA synthetase is apparently absent *in vivo* but essentially normal *in vitro*. It seems reasonable to conclude that lack of repression *in vivo* is not due to any *irreversible* change in control of gene expression associated with carcinogenesis. It may be that ethionine has some extrahepatic effect (e.g. on heme absorption or removal) such that little heme reaches the liver *in vivo*. This seems unlikely since induction of hepatic TAT by hemin *in vivo* was essentially normal in ethionine-fed rats, and it has been shown that such TAT induction is due, at least in part, to a direct effect of heme on liver cells [34].

The general alternative to an extrahepatic effect of ethionine is that it causes some change *in vivo*, either within or at the cell membrane of liver cells, which is reversed or modified by the procedure of cell isolation or by the conditions of incubation of the isolated cells. Ethionine *in vivo* might, for instance, reduce entry of heme to the liver, although the normal induction of TAT by hemin suggests (but does not prove) that this is not the case. One alternative possibility involves cyclic adenosine-3′, 5′-monophosphate (cAMP). AIA-stimulated induction of ALA synthetase in isolated cells was shown previously to be largely dependent on the presence of cAMP or its dibutyryl derivative [20]; hence, optimal concentrations of dibutyryl cAMP were routinely added to all liver cell suspensions. As a result, intracellular levels of cAMP in isolated-cell experiments are probably relatively high, both compared to normal levels *in vivo*, and regardless of any changes, following ethionine-treatment, in the ability of cells to generate or maintain cyclic AMP. Conversely, any ethionine-induced change in liver cells involving reduced cAMP levels or reduced sensitivity to the nucleotide might be reserved under the conditions of our liver cell experiments. Whether an ethionine-induced change of this type could account for loss of heme (and cholesterol?) feedback effects is uncertain, although the nucleotide is at least implicated in regulation of both ALA synthetase [20] and β-hydroxy-β-methylglutaryl coenzyme A reductase [35]. It is also known to have marked effects on the state of "differentiation" of a variety of neoplastic cells [36].

Whatever the ultimate explanation, there seems to be little doubt that further studies on the differences between defective vival but effective vitral feedback controls in carcinogen-treated liver will be important to an understanding of the early effects of carcinogens and the relationship of these effects to carcinogenesis.

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REFERENCES

1. V. R. Potter, Biochemical perspectives in cancer research. *Cancer Res.* **24,** 1085 (1964).
2. M. D. Siperstein, Regulation of cholesterol biosynthesis in normal and malignant tissues. *Curr. Top. Cell Reg.* **2,** 65 (1970).
3. J. R. Sabine, Defective control of lipid biosynthesis in cancerous and precancerous liver. *Progr. biochem. Pharmacol.* **10,** In press.
4. F. de Matteis, Disturbances of liver porphyrin metabolism caused by drugs. *Pharmacol. Rev.* **19,** 523 (1967).
5. A. Kappas, R. D. Levere and S. Granick, The regulation of porphyrin and heme synthesis. *Semin. in Hematol.* **5,** 323 (1968).
6. S. Granick and S. Sassa, δ-Aminolevulinic acid synthetase and the control of heme and chlorophyll synthesis, In *Metabolic Pathways*, (Edited by H. J. Vogel), Vol. 5, p. 77. Academic Press, New York (1971).

7. D. P. TSCHUDY and H. L. BONKOWSKY, Experimental porphyria. *Fed. Proc.* **31,** 147 (1972).
8. H. S. MARVER, The role of heme in the synthesis and repression of microsomal protein, In *Microsomes and Drug Oxidations* (Edited by J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 495. Academic Press, New York (1969).
9. N. HAYASHI, Y. KURASHIMA and G. KIKUCHI, Mechanism of allylisopropylacetamide-induced increase of δ-aminolevulinate synthetase in liver mitochondria V. Mechanism of regulation by hemin of the level of δ-aminolevulinate synthetase in rat liver mitochondria. *Arch. Biochem. Biophys.* **148,** 10 (1972).
10. S. L. M. GIBSON and A. GOLDBERG, Defects in haem synthesis in mammalian tissues in experimental lead poisoning and experimental porphyria. *Clin Sci.* **38,** 63 (1970).
11. R. BARNES, M. S. JONES, O. T. G. JONES and R. J. PORRA, Ferrochelatase and δ-aminolaevulate synthetase in brain, heart, kidney and liver of porphyric rats. *Biochem. J.* **124,** 633 (1971).
12. J. S. WOODS and R. L. DIXON, Studies of the perinatal differences in the activity of hepatic δ-aminolevulinic acid synthetase. *Biochem. Pharmacol.* **21,** 1735 (1972).
13. J. S. WOODS and R. L. DIXON, Perinatal differences in delta-aminolevulinic acid synthetase activity. *Life Sci.* **9,** 711 (1972).
14. C. S. SONG, J. W. SINGER, R. D. LEVERE, D. F. HARRIS and A. KAPPAS, Developmental and gestational influences on drug induction of δ-aminolevulinic acid (ALA) synthetase in rat liver. *J. Lab. clin. Med.* **72,** 1019 (1968).
15. 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.* **37,** 1702 (1967).
16. B. J. HORTON and J. R. SABINE, Metabolic controls in precancerous liver—IV. Loss of feedback control of cholesterol synthesis and impaired cholesterol uptake in ethionine-fed rats. *Europ. J. Cancer* **9,** 11 (1973).
17. D. P. TSCHUDY, F. H. WELLAND, A. COLLINS and G. HUNTER, The effect of carbohydrate feeding on the induction of δ-aminolevulinic acid synthetase. *Metabolism* **13,** 396 (1964).
18. H. S. MARVER, A. COLLINS, D. P. TSCHUDY and M. RECHEIGL, δ-Aminolevulinic acid synthetase—II. Induction in rat liver. *J. biol. Chem.* **241,** 4323 (1966).
19. M. N. BERRY and D. S. FRIEND, High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43,** 506 (1969).
20. A. M. EDWARDS and W. H. ELLIOTT, Induction of δ-aminolevulinic acid synthetase in isolated rat liver cell suspensions: adenosine-3′,5′-monophosphate dependence of induction by drugs. *J. biol. Chem.* **249,** 851 (1974).
21. M. J. WHITING and W. H. ELLIOTT, Purification and properties of solubilised mitochondrial δ-aminolevulinic acid synthetase and comparison with the cytosol enzyme. *J. biol. Chem.* **247,** 6818 (1972).
22. S. GRANICK, The induction *in vitro* of the synthesis of δ-aminolevulinic acid synthetase in chemical porphyria: A response to certain drugs, sex hormones, and foreign chemicals. *J. biol. Chem.* **241,** 1359 (1966).
23. F. ROSEN, H. R. HARDING, R. J. MILHOLLAND and C. A. NICHOL, Glucocorticoids and transaminase activity—VI. Comparison of the adaptive increases of alanine and tyrosine-α-ketoglutarate transaminases. *J. biol. Chem.* **238,** 3725 (1963).
24. 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).
25. B. J. HORTON, J. D. HORTON and J. R. SABINE, Repeated estimation of liver cholesterol synthesis *in vivo*, using the inhibitor AY-9944. *Biochim. biophys. Acta* **239,** 475 (1971).
26. N. HAYASHI, B. YODA and G. KIKUCHI, Mechanism of allyl-isopropylacetamide-induced increase of δ-aminolevulinate synthetase in liver mitochondria—IV. Accumulation of the enzyme in the soluble fraction of rat liver. *Arch. Biochem. Biophys.* **131,** 83 (1969).

27. 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).
28. H. L. Bonkowsky and D. P. Tschudy, Control of δ-aminolevulinic acid synthetase (ALAS), ALA-dehydrase (ALAD), and tyrosine aminotransferase (TAT) in tumors and livers of tumor-bearing rats. *Proc. Amer. Ass. Cancer Res.* **12,** 8 (1971).
29. H. L. Bonkowsky, D. P. Tschudy, A. Collins and J. M. Doherty, Control of δ-aminolevulinic acid synthetase and tyrosine aminotransferase in tumors and livers of tumor-bearing rats. *J. natl. Cancer Inst.* **50,** 1215 (1973).
30. M. Higgins and H. Rudney, Regulation of rat liver β-hydroxy-β-methylglutaryl–CoA reductase activity by cholesterol. *Nature New Biol.* **246,** 60 (1973).
31. 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).
32. J. A. Watson, Regulation of lipid metabolism in *in vitro* cultured minimal deviation hepatoma 7288C. *Lipids* **7,** 146 (1972).
33. J. A. Watson, Regulation of cholesterol synthesis in HTC cells (minimal deviation hepatoma 7288C). In *Tumor Lipids: Biochemistry and Metabolism.* Edited by R. Wood, p. 34. A.O.C.S. Champaign (1973).
34. A. M. Edwards, Induction by heme of hepatic tyrosine aminotransferase (TAT) *in vivo* and in isolated liver cells. *Proc. Aust. Biochem. Soc.* **6,** 64 (1973).
35. L. A. Bricker and G. S. Levey, Autonomous cholesterol and fatty acid synthesis in hepatomas: deletion of the adenosine 3′,5′-monophosphate control mechanism of normal liver. *Biochem. biophys. Res. Comm.* **48,** 362 (1972).
36. R. van Wijk, W. D. Wicks and K. Clay, Effects of derivatives of cyclic 3′,5′-adenosine monophosphate on the growth, morphology and gene expression of hepatoma cells in culture. *Cancer Res.* **32,** 1905 (1972).

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Cytostatic Activity of Chlamydocin, a Rapidly Inactivated Cyclic Tetrapeptide

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Abstract—*Chlamydocin is a cyclic tetrapeptide with an epoxi group which was isolated from cultures of* Diheterospora chlamydosporia. *In cultures of P-815 mouse mastocytoma cells, the coumpound has the highest cytostatic activity found so far in our laboratory (higher than actinomycin D, amethopterin, colchicine, vincristine and others). Toxicity and oncostatic activity in animals upon drug injection is comparatively low, probably due to the fact that the drug is inactivated rapidly in blood (half life about 2·5 min). The chlamydocin-inactivating capacity of serum is lost upon heating for 30 min at 56°C. When given as continuous or interrupted intravenous or intra-arterial infusion, chlamydocin is much more toxic in rats than by injection. Inhibition of growth of the Walker tumor is somewhat better by infusion than by injection and is obtained with much lower doses.*

INTRODUCTION

IN 1967, WE found culture filtrates of *Diheterospora chlamydosporia* to inhibit multiplication of P-815 mouse mastocytoma cells *in vitro*. Purification of the active principle, guided by determination of cytostatic activity, led to the isolation of a lipophilic compound. This compound was then found to be a cyclic tetrapeptide (Fig. 1) consisting of proline, phenylalanine, α-aminoisobutyric acid, and 2-amino-8-oxo-9,10-epoxi-decanoic acid [1].

The high cytostatic activity *in vitro* of the tetrapeptide, designated chlamydocin, combined with a comparatively low toxicity in animals, stimulated us to further investigate the biological effects of this compound in tissue culture and in animals. In this report, results of some of these experiments are presented, pertaining to growth inhibition in tissue culture, toxicity, inactivation of the drug in blood, and tumor inhibition in animals, particularly upon intra-arterial application.

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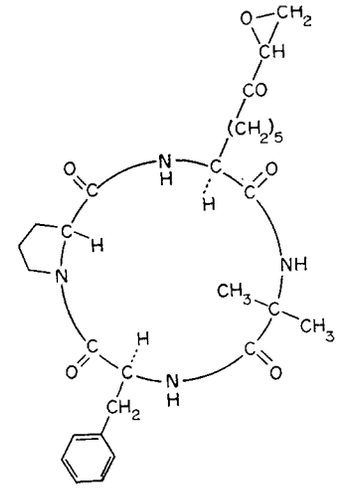


Fig. 1. Structure of chlamydocin.

MATERIAL AND METHODS

Chlamydocin, isolated by our Chemical Research Department from cultures of *Diheterospora chlamydosporia* [1], was dissolved for *in vitro* experiments in a mixture of 9 parts

DMSO (dimethylsulfoxide) and 1 part Tween 80 (0,1 ml of this solvent mixture per mg drug); water or salt solution was then added to obtain the desired concentrations. For animal experiments, the compound was dissolved in a small amount of DMSO, then Tween 80 was added (1·5 or twice as much as DMSO), then water to obtain the desired concentration for injection or infusion.

The method of culturing P-815 mastocytoma cells of the mouse has been described previously [2]. Cells were counted in the Microcellcounter (Toa Medical Electronics Co. Ltd., Kobe, Japan).

For evaluation of inhibition of tumor growth (or increase of life span, ILS) by drug injections, standard methods were used (essentially those used by the CCNSC of the Natl. Cancer Inst., USA [3], as described earlier [4]. Drug was given by the i.p. route except in the cases mentioned in footnote of Table 2.

For drug inactivation studies in biological fluids, drug solution (0·2 ml) was added to 1·8 ml of the respective fluid in a test tube, the mixture incubated for different time intervals in a water bath at 37°C and the amount of remaining active drug measured by determining the cytostatic ED_{50} in cultures of P-815 mastocytoma cells. With serum–drug mixtures, different dilutions (in Hanks' solution) of the incubated mixture were added to the cell cultures, in parallel with known concentrations of fresh drug solutions. The percentage of inactivation or the biological half life was then calculated from the difference between the two ED_{50}. When inactivation was measured in blood, care was taken to add the drug solution immediately after collecting the blood in a heparinized syringe or container and to start incubation at 37°C without delay; after incubation, the blood was cooled immediately in ice water, centrifuged, and the supernatant plasma used for bioassay ("direct method").

In some experiments with rabbit blood, incubation of drug in blood was not stopped by cooling, but by adding to the blood–drug mixture ice-cold Hanks' solution (18 ml Hanks' solution for 2 ml mixture); after that, the diluted blood was shaken immediately with 20 ml chloroform and the chloroform separated in a separatory funnel; the remaining aqueous phase was again extracted with some chloroform and the two batches of chloroform mixed. The chloroform containing the drug was then centrifuged to remove undissolved material (previous experiments had shown that chlamydocin can be extracted from aqueous solutions quantitatively with chloroform). Chloroform was then evaporated in a vacuum at 37°C, the remaining drug dissolved in DMSO and bioassayed as described above.

For tumor inhibition experiments using infusion instead of injection treatment with drug, Wistar rats, 200 g, were housed singly. Walker carcinosarcoma (0·5 ml ascitic fluid) was implanted i.m. into the right calf muscle on day 0. On day 1, a fine polyethylene catheter (Portex Ltd., Hythe, England) was inserted either into the right jugular vein or into the descending aorta (its tip being above the bifurcation of the iliac arteries). The catheter (No. PP50) was fixed in the jugular vein by a ligature around the vein and the distal part of the vein tied off. In the aorta, the catheter No. PP25 was inserted through an incision below the renal artery and fixed to the underlying muscle with a surgical thread. In both cases, the point of exit of the catheter was through the skin at the top of the neck where the tubing was again fixed to the muscle. Before insertion, the catheters were filled with heparin solution. The catheter, protected by flexible metal tubing (attached to the rat by a saddle, modified model 1602, Lehigh Valley Electronics, Fogelsville, Pa.), was connected to a syringe through a cannular feedthrough swivel (model 1603, Lehigh Valley Electronics). This allowed the animal to move and turn freely in its cage. The syringe was operated by an infusion pump (Unita I, B. Braun, Melsungen, Germany) which was set in motion and stopped by an electric timer. Drug concentration in the syringe was adjusted to have the amount to be given per day dissolved in a volume of 0·9–2·7 ml per animal.

RESULTS

1. *Cytostatic activity* in vitro

Chlamydocin was tested for inhibition of multiplication of P-815 mastocytoma cells of the mouse. With an incubation time of 40 hr, the mean concentration inhibiting cell proliferation by 50% (ED_{50}) in 13 experiments was found to be 0·36 (±0·07 S.E.M.) ng/ml, that is $10^{-9{\cdot}44}$ g/ml. Another cell line, C6 (glial tumor of the rat), was of equal sensitivity as P-815 cells to chlamydocin, while HeLa and L cells required higher concentrations for inhibition of proliferation.

The slope of the curve relating log drug concentration to inhibition of cell proliferation is not very steep (Fig. 2). The fact that con-

centrations about $30 \times ED_{50}$ do not diminish the original cell number indicates that treatment with chlamydocin does not lead rapidly to cell lysis.

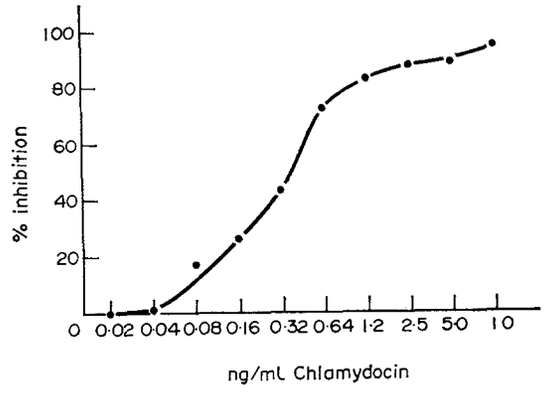


Fig. 2. Inhibition of multiplication of P-815 mastocytoma cells in vitro. *Incubation time: 40 hr.*

2. *Toxicity*

In comparison with its strong cytostatic activity *in vitro*, doses of chlamydocin leading to death of animals are unexpectedly high (Table 1); mice and rats survive single i.v. doses of 160 and 50 mg/kg, respectively. It can also be seen from Table 1 that rats are considerably more sensitive to chlamydocin than mice, particularly when the drug is given orally. Furthermore, there seems to be little cumulation of toxicity since LD_{50} for daily i.v. application for 6 days is similar to the LD_{50} for single application.

Table 1. Acute and subacute toxicity of chlamydocin in mice and rats

Animal	Dosage schedule	Route	LD_{50} mg/kg per inj.
Mouse	Once	i.v.	226
Mouse	Once	p.o.	> 850
Mouse	Daily, 6×	i.v.	160
Rat	Once	i.v.	66
Rat	Once	p.o.	141
Rat	Daily, 6×	i.v.	80

LD_{50}, the 50% lethal dose, was calculated according to Reed and Muench [5].

3. *Tumor inhibition, treatment by injection*

Chlamydocin, injected i.p., was tested for its effect on the survival time (ILS) of mice inoculated with L-1210 (lymphoid), P-815 (mastocytoma) and P-1534 (lymphoid) leukemias. It is evident from the results (Table 2) that this drug produces only very moderate ILS if at all. Particularly surprising was the poor response of P-815 *in vivo*, a tumor cell which is inhibited *in vitro* by very low concentrations of drug (see above); in mice inoculated with this tumor, the only effect was a considerable reduction of the spleen weight of the treated animals as compared to controls.

Growth of the solid sarcoma 180 likewise was inhibited to a very limited degree only by chlamydocin. Somewhat more consistent results were obtained with daily treatment of rats inoculated with the Walker carcinosarcoma; but here too, the mean per cent inhibition of the tumor weight was by no means impressive (Table 2).

The only tumor showing a good response to chlamydocin was the Ehrlich ascites tumor (Table 2), a neoplasm in which a strong reduction of the number of tumor cells in the peritoneal cavity (total packed cell volume, the parameter used in our experiment) is easily obtained by many drugs when they are injected into the ascitic fluid.

It was striking that in a number of experiments (more in mice than in rats) the dose dependency of the response was poor or lacking, i.e. higher doses often produced less tumor inhibition than lower ones, even when the higher doses were not toxic as judged from mortality or body weight or spleen weight (the latter in L-1210).

4. *Drug inactivation in blood and serum*

The discrepancy between the high *in vitro* cytostatic activity of chlamydocin and the comparatively low toxicity and low oncostatic activity in animals led to the hypothesis of a very rapid disappearance from the body of the drug or of its activity. This hypothesis was supported by the results of a preliminary experiment in which it was found that cytostatic activity of the serum of rats which had received 30 min previously 50 mg/kg of chlamydocin i.v., corresponded to a drug concentration of only 14 µg/l. Assuming an even distribution of drug in the body, this low activity would mean that active drug had disappeared with a half life of 2·5 min. Since such a rapid disappearance could not be due to excretion, either sequestration of chlamydocin in some part of the body or rapid inactivation of the drug had to be assumed. The latter possibility was then investigated further in *in vitro* experiments.

Chlamydocin was added to fresh or inactivated serum or to fresh heparinized blood

Table 2. Antitumour effects of chlamydocin, treatment by injection

Tumor	Host*	Treatment	Dose range tested (mg/kg per inj.)	Optimal ILS (%)	Optimal tumor weight reduction (%)	No. of exp.
L-1210	MF	Once	50–400	19†	—	2
10^5 i.p.		Daily	20–160	49‡	—	1
		Twice weekly	20–200	29†	—	2
10^6 s.c.		Daily	20–160	7	—	1
P-815 i.p.	DBA/2	Daily	20–160	10	—	1
P-1534 s.c.	DBA/2	Daily	20–160	0	—	1
Ehrlich Ascites	SIM	Daily	20–160	—	93	1
Sarcoma 180 s.c.	MF	Once	50–400	—	34	1
		Daily	20–320	—	46†	2
Walker carino-sarcoma s.c.	Wistar rat	Once	40–320	—	0	1
		Daily	10–120	—	57§	5
		Twice weekly	10–120	—	45†	2

*MF = (MF2 × DBA/2) F_1 mice; SIM = Swiss mice from closed colony.
†Mean optimal value of two experiments, both of which showed a poor dose dependency.
‡No dose dependency.
§Mean of the optimal value of 3 experiments with i.p. application; range: 24–69%. With i.v. treatment, the effect was negligible and not dose dependent; with p.o. treatment, the lowest dose producing an antitumor effect was toxic.

and the remaining cytostatic activity determined by bioassay after incubation for different time intervals at 37°C (see methods). The results are given in Table 3. It will be seen that the hypothesis of rapid drug inactivation was confirmed. While no or very little loss of activity occurs upon incubation in rat or horse serum inactivated at 56°C for 30 min, there is a fairly rapid disappearance of activity when chlamydocin is incubated in freshly prepared rat serum, half life being 8·7 min. Even more rapid is the decrease of cytostatic activity of the drug when it is added to whole blood; half lives were between 0·68 and 3·6 min. The values of between 2·1 and 3·6 min, obtained for rabbit blood with the chloroform extraction method (Table 3) are probably the most reliable ones. They are in good agreement with the half life of 2·5 min calculated for *in vivo*-inactivation in the rat (see above). The rate of inactivation of chlamydocin in blood does not seem to vary much with the species (rat, rabbit, man) from which the blood is taken.

Since oncostatic drugs with a short biological half life in blood may be suitable for use by infusion, particularly by the intra-arterial route, a number of experiments were done with drug application by infusion.

5. *Tumour inhibition, treatment by infusion*

Groups of 3–6 rats, implanted i.m. with Walker carcinosarcoma, were treated by i.a. or i.v. infusion with different doses of chlamydocin and with different dosage schedules. Control animals were infused with the solvent, using the same schedule. The treatment period was 96 hr (days 4–8 after tumor implantation). The animals were then killed and the percentage of inhibition of tumor growth was calcu-

Table 3. Inactivation of chlamydocin (cytostatic effect) in blood or serum

Fluid	Time* interval (min)	Drug concentration (mg/l)	Biol. half life (min)
rat serum, fresh	0–30	1	8·7
rat serum, inact.†	0–30	1	> 100
rat blood	0–2	1	0·68
rat blood	2–8	1	< 1·5
rabbit blood	0–2	1	1·4
rabbit blood	2–8	1	1·8
horse serum, inact.†	0–60	100	> 1000
human blood	0–2	1	1·7
rabbit blood‡	0–5	0·03	2·5
rabbit blood‡	0–5	1	2·1§
rabbit blood‡	0–5	100	3·6§

*Time interval (incubation at 37°C) for which inactivation was measured.
†Inactivated at 56°C for 30 min. In addition, the horse serum used had been stored for several weeks at 4°C.
‡Inactivation determined with chloroform extraction method (see methods).
§Mean values of 2 experiments; the other figures are values of single experiments each.

lated from the tumor weights of control and treated animals, tumor weight being the weight difference between the implanted and the healthy leg of the rat. Mean tumor weight of 13 control groups was 9·09 g (±3·19 g S.D.) with a range of 4·1–11·7 g.

The results of chlamydocin infusion into the aorta of tumor bearing rats are presented in Table 4. In regard to toxicity, it may be noted that, when given once daily i.v. by injection, rats tolerate 6 × 20–40 mg/kg per injection, and 2–6 died with 6 × 80 mg/kg per day. (There was, however, no significant tumor inhibition with this method; see footnote Table 2). Upon i.a. infusion, toxicity of chlamydocin depends much on the time schedule of infusion. The highest dose tolerated when drug is infused continually for 96 hr is 0·5 mg/kg per day, while with short infusion periods (15 min) once a day, at least 50 times more drug is tolerated. Also, when chlamydocin is infused i.a. for periods of 2 hr with intervals of 2 hr, a dose of 1 mg/kg per day is toxic.

Inhibition of the Walker tumor by i.a. drug infusion is clearly superior to the effects observed with injection (compare Tables 2 and 4). While the optimal effect in a single experiment with i.p. injection was 69 % inhibition (with a dose of 120 mg/kg per day), in several experiments with i.a. infusion tumor inhibitions of > 80 % were observed, and in some cases the mean values of 2–3 experiments with the same infusion schedule are > 70 % inhibition (dosage being here always < 1 mg/kg per day, see Table 4).

Toxicity and inhibition of the growth of the Walker tumor with intravenous infusions of chlamydocin is shown in Table 5. Toxicity seems to be somewhat less than with i.a. infusion, particularly in the controls; thus some of the mortality in the experiments with intraaortic infusion may be attribued to the stress of the surgical procedure. However, a dose of 1 mg/kg per day is lethal for all rats by the i.v. route when infused continuously for 96 hr, and is scarcely tolerated when given in cycles of 2 hr infusion/2 hr interval, similar to the situation with i.a. infusion.

Tumor inhibition by i.v. infusion is perhaps slightly inferior to the results obtained with i.a. infusion (Tables 4 and 5). In one experiment, the effects of i.a. and i.v. infusion (0·5 mg/kg per day, infused continuously for 96 hr were tested side by side with 6 rats each; inhibition was 81 % with i.a., and 60 % with i.v. drug application (figures included in Tables 4 and 5). Average tumor inhibition (3 experiments each) with this dose and schedule was, however, equal with both routes, as was also the case with the schedule 8 hr

Table 4. Inhibition of Walker tumor in rats infused intraarterially with chlamydocin

Schedule* hours On	Schedule* hours Off	Dose (mg/kg per day)	Mortality contr.	Mortality treated	% Tumor inhibition	No. exp.
96	0	0·12	1/11	0/6	20	2
96	0	0·25	1/14	1/9	50	3
96	0	0·5	0/11	3/13	74	3
6	2	0·25	0/8	1/6	64	2
6	2	0·5	0/8	2/6	47†	2
8	8	0·5	0/3	0/3	48	1
2	2	0·25	1/6	1/3	49	1
2	2	0·5	2/9	1/6	78	2
2	2	1·0	0/3	2/3	—	1
0·25	23·75	1–24‡	2/26	3/32	60	5

*In these 2 columns, duration of one infusion period is given (1st column), and the number of hours of drug free intervals between infusion periods (infusion pump off; 2nd column). Total treatment time was always 96 hours.

†Result very questionable because of large difference between the results of the 2 experiments.

‡In these 5 experiments with 1, 2, 3, 6, 12 and 24 mg/kg per day respectively, dose dependency of tumor inhibition was poor; the dose of 24 mg/kg per day resulted in the death of 0 out of 6 animals during the experiment, tumor inhibition was 53%.

infusion/8 hr interval. On the other hand, with the schedule 2 hr infusion/2 hr interval, i.a. infusion produced a mean inhibition of 78% with 0·5 mg/kg per day, while i.v. infusion gave 58% inhibition with 0·6 mg/kg per day (2 experiments each).

(Table 1). With many drugs, e.g. amethopterin or mechlorethamine, the dose producing 50% mortality is much higher for single application than for repeated (daily) injection. A third unexpected feature of chlamydocin was its moderate antitumor effect in animals, despite

Table 5. Inhibition of Walker tumor in rats infused intravenously with chlamydocin

Schedule* hours On	Off	Dose (mg/kg per day)	Mortality contr.	treated	% Tumor inhibition	No. exp.
96	0	0·5	0/17	0/15	73	3
96	0	1·0	0/12	9/9	—	2
8	8	0·6	0/6	0/6	49	1
6	6	0·6	0/12	0/9	48	2
6	6	1·0	0/6	0/3	55	1
2	2	0·6	0/12	0/12	58	2
2	2	1·0	0/6	1/4	73	1
2	2	6	0/6	4/4	—	1

*See footnote 1 of Table 4.

DISCUSSION

With an ED_{50} of 0·36 ng/ml, chlamydocin is the most active compound which we have tested so far for inhibition of proliferation of mastocytoma P-815 cells *in vitro*. It is, in this system, slightly more active than verrucarin A [6] and more than 10 times more active than actinomycin D, vinblastine, vincristine, amethopterine, colchicine, mechlorethamine, and others. Its mechanism of action at the biochemical level is unknown. Regarding its structure, the epoxi group of the unorthodox amino acid makes one think of cytostatic active epoxides which are usually considered to act as alkylating agents [7]. It is very probable that biological activity is influenced decisively by the epoxicarbonyl group in the side chain of chlamydocin, since derivatives without this group were inactive; on the other hand, the epoxicarbonyl side chain without attachment to the tetrapeptide ring was without activity as well [1].

The ratio between the acute LD_{50} in mice and ED_{50} in mastocytoma cultures is exceptionally high for chlamydocin, being of the order of 600,000 (226 mg/kg: 0·00036 mg/l). For other drugs, this ratio is much lower, *e.g.*, about 500 for vincristine and about 6000 for amethopterin. Another surprise was the similarity, in rats and mice, of the LD_{50} for single application and for repeated administration the high ratio between toxic dose (LD_{50}) *in vivo* and cytostatic concentration (ED_{50}) *in vitro*.

These 3 "anomalies" of the compound under investigation led us to the hypothesis of rapid inactivation of the drug in animals. A preliminary experiment with rats gave support to this hypothesis by showing a very rapid disappearance of cytostatic activity in the blood serum after injection of chlamydocin. When the biological half life of the drug *in vivo* found in this experiment is compared to the half life found upon *in vitro* incubation in blood, the values are found to be very similar. This could be interpreted to mean that, *in vivo*, most of the inactivation process occurs in the blood. Biological half lives determined with the chloroform extraction method are somewhat longer than those found with the "direct" method. This difference can be explained by assuming that drug inactivation is not completely arrested by cooling the drug–blood mixture during preparation of the plasma for bioassay in the "direct" method, while with the chloroform extraction method, an immediate and complete stop of inactivation may be expected (determination of the cytostatic activity in blood requires removal of the blood cells because they would render bioassay impossible). Thus, the half life values obtained with the "direct" method are probably somewhat too low.

Fresh rat serum also inactivates chlamydocin

with a relatively short half life. If, however, the serum is heated for 30 min to 56°C before adding the drug, there is practically no more loss of cytostatic activity upon incubation at 37°C for half an hour. Inactivated horse serum likewise does not abolish the cytostatic activity of the peptide. It may therefore be concluded that on one hand, chlamydocin is unstable in blood, and on the other hand, the inactivating capacity of the blood or serum is quite unstable as well, being rapidly lost at a temperature of 56°C. This instability of inactivating power may also be the reason for the slower inactivation in serum as compared to blood, since during preparation of the serum it may loose some of its inactivating effect. The possibility that the blood cells contribute to chlamydocin inactivation must also be considered.

No attempt was made to identify the labile inactivating agent in blood or serum. That inactivation has nothing to do with drug binding to serum proteins is obvious because of the lack of inactivating capacity in heated (56°C) serum and because the inactivating process requires time. Besides that, it was found by ultrafiltration that, at a concentration of 1·0–10 μg/ml, chlamydocin is bound less than 60 % to the proteins of horse or human serum (unpublished observations).

Somewhat disappointing were the results of the infusion experiments. It had been expected that chlamydocin would be better tolerated when given by i.a. infusion than when injected i.v. because some of the drug would be inactivated before reaching the critical tissues after intraarterial infusion. It turned out, however, that the drug is much more toxic upon slow infusion than upon injection or rapid infusion. This is valid for the i.a. as well as for the i.v. route. Probably the half life of chlamydocin in the body is still too long in comparison to the circulation time of the rat. With a rapidly hydrolyzed alkylating agent (CB 1850, half life 4·8 sec), single i.a. injections produced cures of animals inoculated with the Walker carcinosarcoma [8], a tumor known to be particularly sensitive to many alkylating agents [9]. (Chlamydocin produced only slight tumor inhibition in an exploratory experiment when injected once i.a.). Nevertheless, even though not quite as high as expected, tumor inhibition by chlamydocin is better and more consistent with infusion than with injection despite the fact that daily or total dosages are much lower in the infusion experiments. It seems possible that prolonged contact of the drug with the cells is necessary to produce a marked growth inhibition, and that the drug concentration producing this effect may be very low.

The maximal tolerated dose, when infused continuously (i.v. or i.a.) for 96 hr, is 0·5 mg/kg per day (see Tables 4 and 5), i.e. about 70 ng/200 g rat/min. Within the half life of the drug of approximately 2·5 min, a rat thus receives, with this schedule, 175 ng chlamydocin. The maximal concentration of the compound in the animal will then be 252 ng/200 g [10]* or 1·26 ng/g, a concentration which, *in vitro*, inhibits proliferation of P-815 mastocytoma cells by about 85 % (when 1 g of body weight is assumed to be equivalent to 1 ml of culture medium in this respect; see Fig. 2). Ninety-nine per cent of this maximal concentration will be reached within about 17 min (about 6·6 half life periods). Apparently the animal can recover from the effects of infused chlamydocin, since short infusion periods interspaced by long intervals allow higher doses to be given without lethality. Probably for the same reason, equitoxic doses are much higher when given by injection than by (slow) infusion.

The system used in these experiments to demonstrate superiority of the intraarterial route for the prolonged application of a rapidly inactivated cytostatic drug is far from ideal: the rat aorta is small in comparison to the catheter and relatively little blood can pass besides the catheter; furthermore, a comparatively large mass of normal tissues besides the tumor is irrigated by the iliac arteries (that the tumor is reached directly by a drug infused into the aorta descendens was ascertained by immediate coloration of the tumor after infusing Evans' blue). It would therefore seem more appropriate to use larger animals with a tumor located at a site nourished by an accessible and large artery. Our attempts to develop such a system (brain tumors induced by the Schmidt-Ruppin strain of Rous sarcoma virus† in dogs [12]; the brain was chosen because of the lipophilic nature of chlamydocin) were, for technical reasons, not successful. Perhaps the development of such a system, together with optimalization of dosage schedule, may bring about better results of tumor inhibition with i.a. application of chlamydocin than were obtained in our model.

*A formula equivalent to that of Teorell has been developed by Augsberger, expressing the relationship between infusion rate, amount of active drug in the organism and elimination directly in terms of administered dose and half life; [11].

†Generously supplied by Dr. K. Schmidt-Ruppin, Ciba-Geigy Ltd., Basel.

REFERENCES

1. A. Closse und R. Huguenin, Isolierung und Strukturaufklärung von Chlamydocin. *Helv. chim, Acta* **57,** 533 (1974).
2. H. Stähelin, A simple quantitative test for cytostatic agents using non-adhering cells *in vitro*. *Med. exp.* **7,** 92 (1962).
3. CCNSC, Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother. Rep.* **25,** 1 (1962).
4. H. Stähelin, 4'-Demethylepipodophyllotoxin thenylidene glucoside (VM 26), a podophyllum compound with a new mechanism of action. *Europ. J. Cancer* **6,** 303 (1970).
5. L. J. Reed and H. Muench, A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27,** 493 (1938).
6. M. Rüsch und H. Stähelin, Ueber einige biologische Wirkungen des Cytostaticum Verrucarin A. *Arzneitmittel-Forsch.* **15,** 893 (1965).
7. M. Ochoa, Jr. and E. Hirschberg, Alkylating agents. In *Experimental Chemotherapy*. (Edited by R. J. Schnitzer and F. Hawking) Vol. 5, p. 1, Academic Press, New York (1967).
8. L. M. Cobb, Chemotherapeutic activity and pathological effects of an alkylating agent of short half-life designed for intra-arterial infusion (CB 1850). *Int. J. Cancer* **2,** 5 (1967).
9. L. H. Schmidt, R. Fradkin, R. Sullivan and A. Flowers, Comparative pharmacology of alkylating agents. *Cancer Chemother. Rep.*, Suppl. 2, Part 1, p. 1 (1965).
10. T. Teorell, Kinetics of distribution of substances administered to the body. II. The intravascular modes of administration. *Arch. int. Pharmacodyn.* **57,** 226 (1937).
11. K. Saameli and T. K. A. B. Eskes, Bradykinin and cardio-vascular system: estimation of half-life. *Amer. J. Physiol.* **203,** 261 (1962).
12. D. D. Bigner, N. A. Vick, J. P. Kvedar, M. S. Mahaley, Jr., and E. D. Day, Virus-cell relationships in dog brain tumors induced with Schmidt-Ruppin Rous sarcoma virus. *Progr. exp. Tumor Res.* **17,** 40 (1972).

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In Vitro Drug Selection in Antineoplastic Chemotherapy*

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Abstract—*A method has been developed which yields multiple replicate cultures of tumour cells from a single biopsy specimen. The technique has many advantages over previously devised methods. These advantages include ease of repeated microscopic examination without disturbance of cultures and* in vitro *testing of drug effects including the possibility of detection of dose–response relationships; drug combinations can readily be tested serially or concurrently. It is hoped that despite the inherent limitations of any* in vitro *system this promising method may offer a guide to drugs to be avoided because of absence of response* in vitro *as well as a positive indication of potentially useful drugs. The risks, delays, and undesirable consequences of such trials* in vivo *are obvious deficiences of current practice in cancer chemotherapy. Clinical evaluation of the new method of testing will necessarily be a lengthy procedure.*

INTRODUCTION

IMPROVED results in the treatment of leukemias and lymphomas by the use of combinations of cytotoxic drugs have raised the possibility of similar improvements in the treatment of other tumours by the judicious use of selected drugs in combination. Accordingly, there is a new interest in drug selection *in vitro*, further stimulated by the continuing development of new drugs and some improvements in cell culture techniques [1, 2, 4–8, 10, 11, 15, 16]. These techniques have recently been extended to include studies of estrogen binding [14] and prolactin dependence [13] in breast cancer, indicating further potential of the methods. At present, the apparent uniqueness of each tumour and the unpredictability of response result in unnecessary and often hazardous *in vivo* trials in the practice of cancer chemotherapy. Most *in vitro* aids to drug selection which have been proposed are complex, difficult to standardize and do not provide a sufficient number of replicate cultures. Many aliquots of the same cells are essential if a number of drugs is to be tested in a quantitative way so as to yield some indication of dose–response relationships. The method described here offers some advantages in these particular respects.

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MATERIAL AND METHODS

During 1972, biopsies from 102 neoplasms were processed either within a few hours, often less than 1 hr, of operation, or after overnight storage at 4°C in medium 199 from Commonwealth Serum Laboratories (CSL). After trial of several methods of achieving multiple short-term cultures suitable for drug testing, the following procedure was developed for use with any tumour from which a specimen of adequate cellularity is obtained. Thus far, the method has proved satisfactory with carcinoma of ovary, anaplastic carcinoma of gastro-intestinal tract, metastatic carcinoma

in bone, carcinoma of kidney, carcinoma of lung and a single teratoblastoma. Success has not yet been achieved with scirrhous carcinoma of breast.

1. After repeated washing in phosphate-buffered saline (PBS, Dulbecco A), tumour tissue was chopped into 2- to 3-mm pieces. When sufficient tissue was received, a few portions were set aside for explant and organ cultures and sometimes for cytogenetic studies.

2. Fragments remaining were transferred to a conical flask containing a magnetic follower, washed again in PBS if necessary and then covered with 0·25% trypsin (CSL) in PBS warmed to 37°C. The flask was placed on an unheated magnetic stirrer for 5 to 10 min. The resulting cell suspension was transferred to centrifuge tubes containing 0·5 ml of serum, preferably autologous, to neutralize the trypsin. Fresh trypsin was added to the fragments remaining in the flask and the sequence was repeated several times.

3. Cells in the suspension thus accumulated were centrifuged to yield a pellet. The supernatant was removed and the cells resuspended in complete culture medium. That used throughout the development of this method has been: 80% medium 199 (CSL), 15% autologous serum when available or calf serum (CSL), 5% lactalbumin hydrolysate (CSL), penicillin 50 IU/ml, streptomycin 50 μg/ml (CSL), and amphotericin B (Squibb) 2·5 μg/ml.

4. The suspension was examined with regard to viability (using Trypan Blue exclusion test where doubtful) and cell concentration. If the suspension was of satisfactory concentration, homogeneous and viable, it was inoculated into at least two Falcon flasks (Tissue Culture Flask 3012) to form monolayers which could subsequently be removed for secondary inoculation into a microplate (*vide infra*). It is impossible to set down rigid criteria for cell concentration or viability in view of the variation in size and cellularity of biopsy specimens: excessive cell concentrations are easily adjusted by dilution but suspensions of low cell concentration and low percentage of viable cells were still used and some formed satisfactory monolayers. A total of 5×10^6 cells (e.g. 10 ml of 5×10^5 cells per ml) is preferred, for each Falcon flask: where fewer cells are available all may be inoculated into a single flask. When additional viable cells are available the opportunity is taken to set up coverslip cultures and primary microplates. For microplate cultures, Falcon plates (Microtest II Tissue Culture Plate 3040 with Microtest Lid 3041) are preferred: the somewhat similar Cooke plates (Microtiter Tissue Culture Plate, M-29 ART with M-42 lid) have proved less satisfactory; each microplate contains 96 numbered wells. Approximately 1000 cells in 0·1 or 0·2 ml medium were inoculated into each well; the total number of wells inoculated was dependent on the amount of suspension available. Where cell numbers permit, a second microplate was set up using $2–5 \times 10^3$ cells per well. It has been noted that a rate of monolayer formation suitable for drug assay occurs at a high cell concentration in some tumours and at a lower cell concentration in others.

Addition of drugs

Drugs and hormones considered most relevant to the tumour under examination were added to the microplate when satisfactory monolayers were established, or in the case of a secondary culture, at 72 hr after subculture [12]. In all cases, drug testing was completed within a maximum period of 30 days from receipt of the biopsy, and usually within 15 days.

Drug concentrations used in the microplate wells were calculated with reference to the experience of other laboratories and the maximum therapeutic dose in relationship to the plasma volume of an average patient. A basic concentration (n) was derived for each drug; this concentration as well as $n/10$ and $10n$ were used, each in triplicate or quadruplicate. The final three concentrations adopted for each drug were as follows:

Actinomycin-D (Merck Sharp & Dohme)
0·0005, 0·005, 0·05 μg/ml

Cyclophosphamide (Mead-Johnson)
40, 400, 4,000 μg/ml

5-fluorouracil (Roche)
1·5, 15, 150 μg/ml

Methotrexate (Lederle)
0·5, 5, 50 μg/ml

Procarbazine (Roche)
0·2, 2·0, 20 μg/ml

Thiotepa (Lederle)
0·03, 0·3, 3·0 μg/ml

Vinblastine (Lilly)
0·01, 0·1, 1·0 μg/ml

Vincristine (Lilly)
0·003, 0·03, 0·3 μg/ml

Pharmacological problems with cyclophosphamide necessitated a modification in concentration as suggested by Dendy *et al.* in 1970 [4].

When a combination of drugs was to be tested, the drugs were used, where possible, at two concentrations:

1. Equal to *n* (see above), (e.g. 5-fluorouracil 15 μg/ml plus cyclophosphamide 400 μg/ml).
2. Equal to *n*/2 for each drug.

Medium was changed in all wells of the microplate at the time of drug addition; the control wells received medium only and test wells received medium containing the selected drug or drugs. A complete medium change was effected at 48 hr.

Assessment of drug effects on tumour growth

Using an inverted microscope, observations were made at 24, 48, 72, and 96 hr after the addition of drugs to assess their effects on the monolayers. The key to the drugs added was usually not available to the person assessing the conditions of the monolayers during the subsequent days.

(a) *Morphological changes.* Cytotoxic effects could be readily noted but attempts to standardize and quantitate the changes proved more difficult. The following scale was developed:

Grade 4: intact monolayer, covering at least three-quarters of the lower surface of the well;

Grade 3: healthy cells covering more than one-half but less than three-quarters of the surface;

Grade 2: healthy cells covering more than one-quarter but less than one-half of the surface;

Grade 1: mainly rounded cells, healthy cells less than one-quarter of the surface;

Grade 0: hardly any surviving cells.

This method gave some measure of dose–related response but proved tedious and was not free of subjective bias. It could prove valuable as a rapid rough guide to drug effects, possibly more precise than most morphological methods currently in use.

(b) *Numerical assessment of cells remaining adherent to the lower surface of the chambers in the microplate.* It is recognized that after the rounding-up of cells, the next degree of cytopathic effect includes detachment of cells from the surface on which the monolayer has formed [1]. Hence, the number of cells remaining adherent or the degree of confluence of the monolayer may be used to assess drug effects. An assessment of the adherence of the monolayer was facilitated by use of an eyepiece graticule depicting the Curtis modification [3] of the Chalkley counting device (G52 Chalkley Point Array, from Graticules Ltd., Kent, England) [1]. The random array of points on the graticule, when placed in the field of view over a monolayer, yields a score of 0–25 when coincidences between points and monolayer are counted (Fig. 1). The 96 wells in a microplate can be assessed within 1 hr. The method is approximate but the degree of accuracy, estimated as ±10% is adequate for the present purpose. An initial count is made prior to the addition of drugs, followed by daily counts for 4 days. Using the mean counts obtained for each drug concentration, graphs may be constructed.

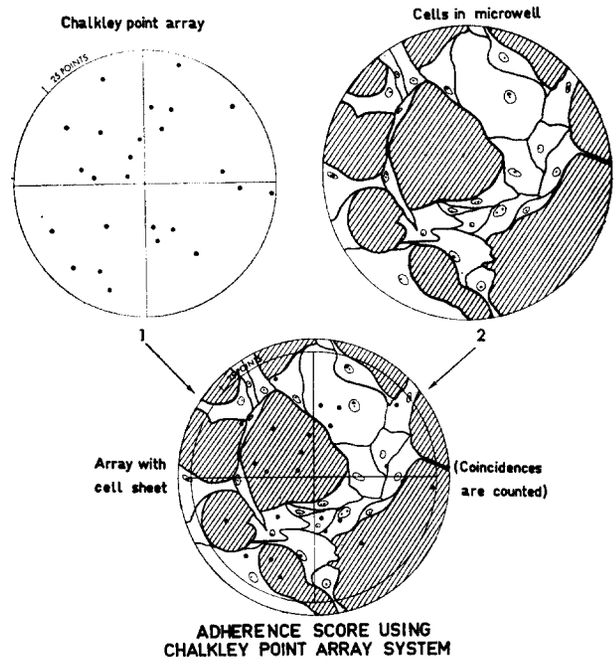


Fig. 1. *Use of Chalkley Point eye-piece Graticule to yield an adherence score.*

An Illustrative Case

A biopsy was taken on November 22, 1972, from an osteolytic lesion of a rib; histopathology suggested either reticulum cell sarcoma or possibly anaplastic carcinoma. Drugs selected for *in vitro* testing in the microplate system were: cyclophosphamide, 5-fluorouracil, methotrexate and procarbazine; as well as cyclophosphamide and fluorouracil and cyclophosphamide and vincristine in combination. Immediate inoculation of a microplate was

undertaken from the primary cell suspension, and a second microplate culture was prepared from a monolayer first grown in a Falcon flask. Assessment of drug effects was made using morphological criteria and the score of adherent cells using the Chalkley point device (Fig. 2, 3). It was found that both microplate cultures gave a similar pattern of drug response: the graphs illustrate the results.

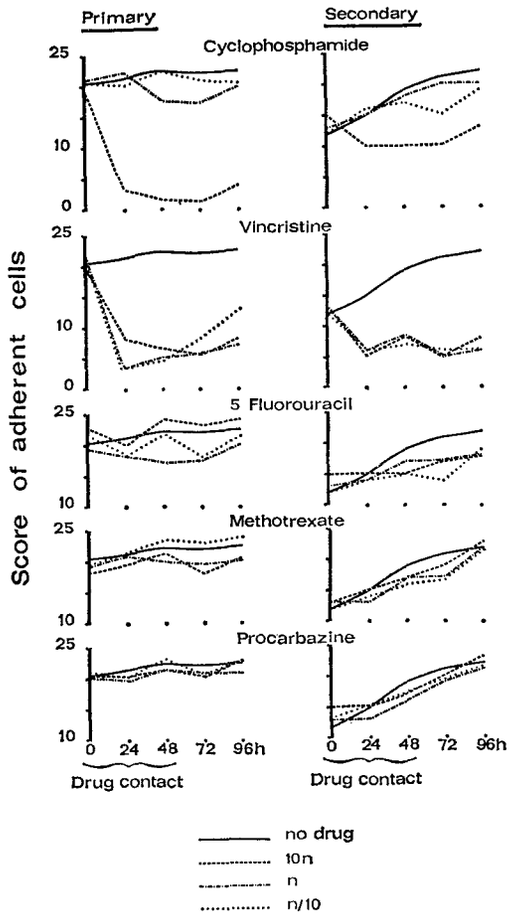


Fig. 2. In vitro *sensitivity in both primary and secondary cultures to antineoplastic drugs, suggesting sensitivity to cyclophosphamide and vincristine, but not to 5-fluorouracil, methotrexate or procarbazine.*

Coverslip cultures were also set up from the suspensions. When monolayers were established, the medium was changed and replaced by fresh medium containingcy clophosphamide, 5-fluorouracil or vincristine. Forty-eight hours later, the medium was replaced by fresh medium without drugs. The coverslip cultures were fixed and stained after a further 48 hr. Representative photographs of the results are presented in Fig. 4.

It will be noted that the cultures were significantly affected by vincristine and cyclophosphamide but not by 5-fluorouracil, methotrexate or procarbazine, at least during the period of observation. The patient was treated with nitrogen mustard, vincristine, procarbazine and prednisolone prior to these results becoming available: response was satisfactory but some vincristine neuropathy was encountered. In view of the *in vitro* results, continuing use of a vinca alkaloid, but not of procarbazine is proposed.

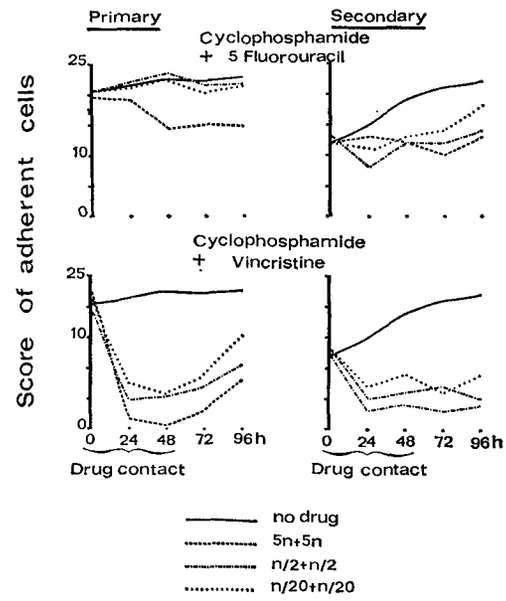


Fig. 3. In vitro *sensitivity to antineoplastic drugs in combination indicating sensitivity to the combination of cyclophosphamide and vincristine.*

DISCUSSION

An ideal method of *in vitro* drug testing would be simple, consistent, statistically acceptable, easily standardized, comparatively cheap, relatively rapid, flexible, capable of taking into account various methods of drug action as well as offering reasonable correlation with the *in vivo* use of the same drug or drugs [9]. No method available meets all these criteria but the micro-method described may offer some advantages over current methods as:

1. Multiple cultures are readily available, allowing sufficient control cultures;
2. Dose-related response can be seen;
3. The drug effects may be observed over a period of time so that rapid cell recovery or prolonged cell damage, or changes in the ecology of the monolayer can be appreciated; observation may continue after removal of drugs;

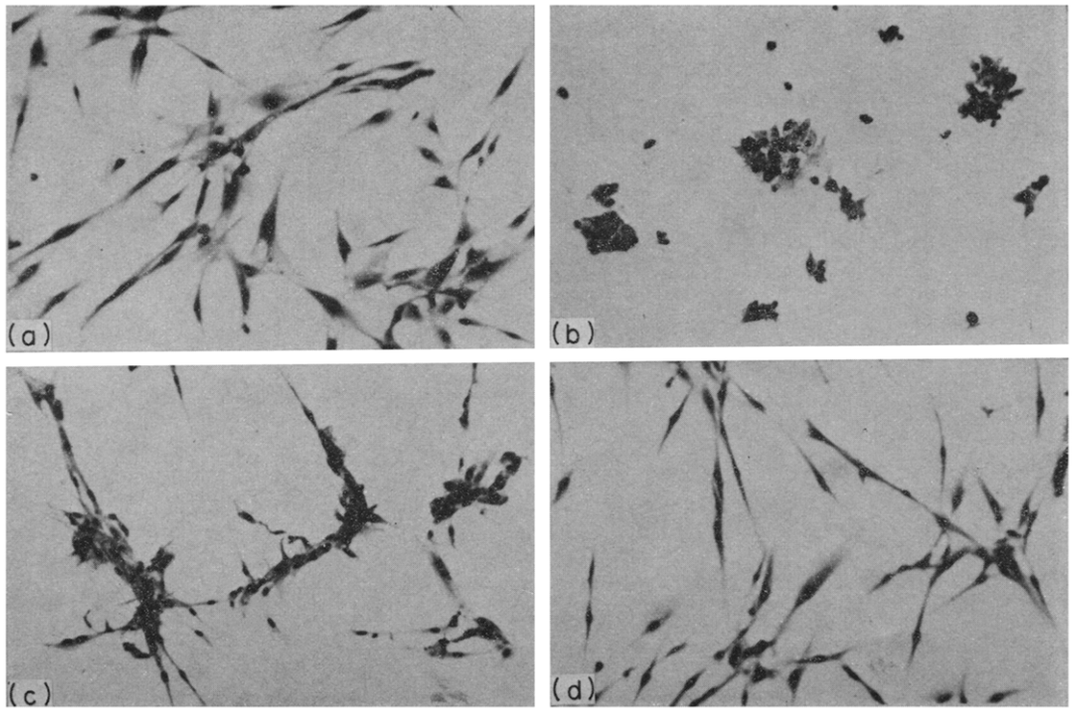


Fig. 4

(to face p. 812)

4. Combinations of drugs can be tested either synchronously or serially;

5. The monolayers in the microwells may be studied in other ways, with or without drug testing. The method may prove adaptable to biochemical or autoradiographic techniques.

Theoretical as well as practical problems remain. The microplate method has not yet proved successful in the case of relatively non-cellular or slow-growing tumours. An important failure so far has been with carcinoma of breast, although this may be related to the structure of the tumour with much fibrous stroma, or to the hormonal requirements of the cells; modifications of the method may overcome the present difficulties. Further, it is not easy to predict success or failure of the method in individual cases—some rapidly-growing monolayers failed to grow when sub-cultured from Falcon flasks onto microplates.

What is the level of *in vivo/in vitro* correlation? This question requires further study with reference to the microplate system. At least a drug which fails to be cytopathic *in vitro* may be avoided *in vivo* if more promising alternatives are available.

Several modifications of the method suggest themselves. The period of drug contact can be varied readily and shorter drug contact times are currently being used. Already the concentrations of drugs being used have been increased by a factor of 10 so that the levels in the medium might reflect the blood levels obtained by the higher doses in current therapeutic use. Drug effects are being sought over a longer period of observation, allowing for the longer cell cycles of many tumours and the varying mechanisms by which the different drugs exert cytopathic effects.

In conclusion, the microplate method, inherently simple in conception and execution, may have considerable potential and would appear to justify further examination and, no doubt, modification. The assessment of the clinical value of the results obtained is likely to be a considerable undertaking in view of case-to-case variations and the optimal period of follow-up.

REFERENCES

1. E. J. Ambrose, R. D. Andrews, D. M. Easty, E. O. Field and J. A. H. Wylie, Drug assays on cultures of human tumour biopsies. *Lancet* **1,** 24 (1962).
2. I. J. Bickis, W. D. Henderson and J. H. Quastel, Biochemical studies on human tumours. II. *In vitro* estimation of individual tumor sensitivity to anti-cancer agents. *Cancer* **19,** 103 (1966).
3. A. S. Curtis, Area and volume measurements by random sampling methods. *Med. biol. Il.* **10,** 261 (1960).
4. P. P. Dendy, G. Bozman and T. K. Wheeler, Screening test for human malignant tumours before chemotherapy. *Lancet* **2,** 68 (1970).
5. P. P. Dendy, V. G. Bozman, T. K. Wheeler and M. P. A. Dawson, An *in vitro* screening test for hormonal and other forms of cytotoxic chemotherapy. In *Some Implications of Steroid Hormones in Cancer.* (Edited by D. C. Williams and M. H. Briggs), p. 107. Heinemann, London (1971).
6. D. M. Easty and J. A. H. Wylie, Screening of 12 gliomata against chemotherapeutic agents *in vitro*. *Brit. med. J.* **1,** 1589 (1963).
7. W. C. Gewant and I. S. Goldenberg, Techniques of human breast neoplasm cell culture. *Europ. surg. Res.* **2,** 392 (1970).
8. J. D. Hurley and L. J. Yount, Selection of anti-cancer drug for palliation using tissue culture sensitivity studies. *Amer. J. Surg.* **109,** 39 (1965).
9. Testing anticancer drugs. *Lancet* **1,** 827 (1972).
10. H. Lazarus, W. Tegeler, H. Mazzone, J. Leroy, B. Boone and G. Foley, Determination of sensitivity of individual biopsy specimens to potential inhibitory agents. Evaluation of some explant culture methods as assay systems. *Cancer Chemother. Rep.* **50,** 543 (1966).
11. H. Limburg and U. Heckmann, Chemotherapy in the treatment of advanced pelvic malignant disease with special reference to ovarian cancer. *J. Obstet. Gynaec. Brit. Commonw.* **75,** 1246 (1968).
12. J. Paul, *Cell and Tissue Culture,* 4th Ed. p. 342. Livingstone, Edinburgh (1970).
13. H. Salih, H. Flax, W. Brander and J. R. Hobbs, Prolactin dependence in human breast cancers. *Lancet* **2,** 1103 (1972).

14. H. Salih, H. Flax and J. R. Hobbs, *In vitro* oestrogen sensitivity of breast cancer tissue as a possible screening method for hormonal treatment. *Lancet* **1,** 1198 (1972).
15. R. Tchao, G. C. Easty, E. J. Ambrose, R. W. Raven and H. J. G. Bloom, Effect of chemotherapeutic agents and hormones on organ cultures of human tumours. *Europ. J. Cancer* **4,** 39 (1968).
16. M. Yarnell, E. J. Ambrose, K. Shepley and R. Tchao, Drug assays on organ cultures of biopsies from human tumours. *Brit. med. J.* **2,** 490 (1964).

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Is There a Factor in Sera From Cancer Patients That Inhibits Lymphocyte Response to Phytohaemagglutinin?

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Abstract—*Lymphocytes from patients with benign breast disease (used as controls), patients with early breast cancer, and patients with advanced breast cancer were stimulated by PHA in the presence of one of five types of serum: (a) autologous serum, (b) AB serum, (c) pooled serum from patients with advanced breast cancer, (d) pooled serum from patients with advanced colon cancer, (e) foetal calf serum.*

Control lymphocytes were significantly inhibited by sera from all of the four non-self groups ($P<0{\cdot}001$), whereas lymphocytes from early breast cancer patients were significantly inhibited by advanced colon cancer serum ($P<0{\cdot}05$), AB serum ($P<0{\cdot}05$), and foetal calf serum ($P<0{\cdot}01$). Lymphocytes from advanced breast cancer patients were inhibited by all the non-self sera but the inhibition was only significant with pooled advanced colon cancer serum ($P<0{\cdot}05$).

The most likely explanation is that the inhibition produced is due to the non-self serum rather than to a specific inhibitory factor. However, it is possible that there may be such a factor in the serum of advanced colon cancer patients.

INTRODUCTION

IT HAS been known for a number of years that the response of lymphocytes from cancer patients to phytohaemagglutinin (PHA) is diminished [1]. This effect could be due either to a defect in the lymphocytes of the cancer patient or to the presence of an inhibitor in the serum. Support for the former concept is provided by the impairment of delayed hypersensitivity reactions found in cancer patients [2, 3]. Evidence for a serum inhibitory factor is provided by several reports of a decrease in PHA-induced stimulation of human lymphocytes in the presence of serum from cancer patients. Whittaker *et al.* [4] described a factor in the serum of patients with advanced breast cancer which inhibited lymphocyte stimulation in both breast cancer and control patients. A factor inhibiting lymphocyte response to PHA has also been demonstrated in the serum of patients with various non-lymphoid malignancies by other workers [5–8]. However, there have also been reports where no inhibitory factor was found in the serum of cancer patients [9, 10].

Al-Sarraf *et al.* [11] found that any allogeneic serum caused a decrease in lymphocyte response to PHA and concluded that the inhibition reported with cancer serum was a non-specific manifestation of this effect. Further conflicting evidence has been reported by McIllmurray *et al.* [12], who found that the PHA response of lymphocytes from healthy individuals was enhanced in the presence of plasma from patients with advanced intestinal cancer.

High serum levels of alpha globulins have been found to inhibit PHA stimulation [13], but Gatti [5] found no correlation between inhibitory activity in cancer serum and alpha globulin levels.

The final conclusion remains uncertain as

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some authors report a specific inhibition due to a factor in cancer serum, some a non-specific inhibition due to any allogeneic serum, some no effect, and some enhancement.

We have attempted to clarify this confused situation by comparing the PHA response of lymphocytes from patients with benign breast lesions, early breast cancer and advanced breast cancer in the presence of five different types of serum:

(*a*) autologous serum;
(*b*) human AB serum;
(*c*) pooled serum from patients with advanced breast cancer;
(*d*) pooled serum from patients with advanced colon cancer;
(*e*) foetal bovine serum.

We considered that by using these different sera, the following questions might be answered:

(i) Does autologous serum give the maximal response?
(ii) Is there a specific inhibitory factor in sera from advanced cancer patients?
(iii) If so, is this factor specific and related to a type of cancer, or present in sera from all patients with cancer?
(iv) Is there a stimulatory factor in sera from patients with advanced cancer of the colon?

MATERIAL AND METHODS

Patients

Lymphocytes for study were obtained from patients admitted for biopsy and treatment of suspected carcinoma of the breast and from patients with local recurrence or metastases. Blood samples were taken prior to commencement of treatment. Histological confirmation of the diagnosis was obtained in all cases.

Patients were divided into three groups, 19 control patients with benign disease on biopsy, 8 patients with "early" disease considered to be potentially curable by surgery, and 14 patients with advanced local or metastatic disease. Using the TNM classification, "early" patients were T1-2, NO-1, MO. Nine of the group with advanced breast cancer had haematogenous dissemination, the remaining 5 having advanced local disease. The age range of the three groups was similar (Table 1).

Sera

Autologous serum was separated from a sample obtained at the same time as blood was taken for lymphocyte culture. AB serum was obtained from a healthy Blood Bank donor. The advanced breast cancer serum pool consisted of sera from 12 patients with metastatic disease. None of these patients was receiving cytotoxic therapy at the time of bleeding and none was included as a donor of the lymphocytes in the advanced breast group. Similarly, serum was obtained from 8 patients with advanced cancer of the colon with either an unresectable local tumour or hepatic metastases. Foetal calf serum was obtained commercially (Tissue Culture Services, Slough, Bucks). All sera were stored in 5 ml aliquots at −70°C prior to use. Sterilisation was performed by membrane filtration.

The alpha-2 globulin levels were measured by cellulose acetate electrophoresis followed by staining with Ponceau S and densitometry. The alpha-2 macroglobulins were measured by radial immuno-diffusion (Baxter plates).

Lymphocyte culture

Lymphocytes were separated from 20 ml of venous blood using the method of Bøyum [14]. The lymphocytes were washed and diluted in TCM199 + 20 % of the various sera for stimulation tests. All cultures were performed in triplicate. The lymphocyte count varied from 2×10^5/ml to 10^6/ml depending on the number of lymphocytes available but was constant for the one patient with all the sera tested. Two μg/ml of purified PHA (Wellcome Laboratories) were added to 1·5 ml cultures in screw-capped pyrex tubes and incubated for 72 hr at 37°C. One hour before harvesting, 3 μCi of tritiated thymidine (specific activity 20 mCi/mM, Radiochemical Centre, Amersham) were added to each culture and the tubes reincubated. The cells were rinsed from the tubes with cold saline, centrifuged, washed again in saline, then once in cold 5 % trichloracetic acid and once in methanol. After drying overnight, the pellets were dissolved in Protosol (NEN Chemicals GmbH, Reading), taken up in scintillation fluid (PPO, dimethyl POPOP and toluene), and counted in an LKB–Wallac liquid scintillation counter. Results were expressed in counts/min.

Statistical analysis

The mean response in each serum for each patient was calculated by subtracting the mean of the unstimulated cultures from that of the stimulated cultures. The logarithms of the means for stimulation in the presence of each serum were then subjected to an analysis of variance in order to perform within-subjects comparisons between sera. Geometric means for each of the sub-groups are shown in Table 1.

Table 1. *Comparison of geometric means of PHA stimulation in* 5 *types of serum*

Patient group	Age range (years)	Autologous	AB	Pooled advanced breast cancer	Pooled advanced colon cancer	Foetal calf serum
Benign breast disease (19)	25–69	53000	21600*	23600*	20000*	16600*
Early breast cancer (8)	29–85	30600	10200†	18100	9400†	6500‡
Advanced breast cancer (14)	32–80	19500	13000	16000	8100†	10000

*$P<0.001$
†$P<0.05$
‡$P<0.01$
} Significance determined in relation to stimulation in autologous serum.

RESULTS

All non-self sera caused depression of PHA stimulation of control lymphocytes (when compared with the values for autologous serum). This depression was highly significant in all four serum groups ($P < 0.001$).

Lymphocytes from patients with early breast cancer were significantly depressed in the presence of foetal calf serum ($P < 0.01$) and in advanced colon cancer serum and AB serum ($P < 0.05$). The depression caused by advanced breast cancer serum in these patients was not statistically significant.

The lymphocyte response to PHA in patients with advanced breast cancer was depressed by all the non-self sera but this depression only reached statistical significance with pooled advanced colon cancer serum ($P < 0.05$).

Of the four types of non-self serum used, pooled advanced colon cancer serum had the most inhibitory effect, causing significant inhibition in all three groups of patients. Foetal calf serum was almost as inhibitory whereas pooled advanced breast cancer serum only caused significant depression of the PHA response in the lymphocytes of control patients.

Only one of the 22 cancer patients stimulated less well in autologous serum than in any other serum. This patient was receiving cortisone acetate 25 mg b.d. and fluodrocortisone 0·1 mg daily following bilateral adrenalectomy, performed six months previously.

The levels of alpha-2 globulins and alpha-2 macroglobulins in the allogeneic sera are shown in Table 2. The alpha-2 macroglobulin levels are within normal limits. However, alpha-2 globulin levels are above the upper limit of normal in both the pooled breast cancer and pooled colon cancer serum.

DISCUSSION

Various causes have been suggested for the depression of PHA response in cancer patients described in earlier reports.

There have been reports of a specific factor directly attributable to the tumour in breast cancer [4] and gastrointestinal cancer [7]. These findings have been interpreted as being related, at least in part, to the immune depression found in patients with advanced cancer. However, Al-Sarraf *et al.* [11] in similar experiments found that inhibition of lymphocyte stimulation by PHA could be induced by the addition of any homologous serum.

We have found that autologous serum gave the best stimulation in all control patients and in all but one of our breast cancer patients. In contrast, Guillou *et al.* [7], using lymphocytes from patients with gastrointestinal cancer, found that stimulation was enhanced in the presence of AB serum compared with autologous serum. Pooled serum from carcinoma of the colon patients was the most inhibitory of all the human sera we tested.

Table 2. *Levels of alpha-2 globulin fractions (g/litre) in sera used*

Serum	Alpha-2 Globulin	Alpha-2 Macroglobulin
AB	5·45	1·6
Pooled breast	10·6	2·0
Pooled colon	10·0	1·6
Normal values	2·5–8·2	1·5–4·2

The inhibition caused by pooled advanced breast cancer serum was no greater than that caused by homologous AB serum. It is likely that this inhibition was the effect of homologous serum as described by Al-Sarraf *et al.* [11] rather than being due to a specific factor related to the patients' tumours.

The inhibition produced by the pooled serum from patients with advanced carcinoma of the colon is unlikely to be due to a specific inhibitory factor, as it did not differ significantly from that produced by the AB serum in any of the three groups of patients. Therefore, we have not produced any definite evidence for the presence of an inhibitory factor in either of the sera from patients with advanced cancer. We have found no evidence of stimulation in the presence of advanced colon cancer serum as described by McIllmurray *et al.* [12].

The serum alpha-2 globulins were elevated above the upper limit of normal in both types of advanced cancer sera. This finding is consistent with that of Cooperband *et al.* [13], but is unlikely to be the sole explanation for the diminution of PHA lymphocyte response, as the AB serum was similarly inhibitory without corresponding elevation of alpha-2 globulin level. The least inhibitory of these three sera was the pooled advanced breast cancer serum which had the highest level of alpha-2 globulin.

Our results favour the concept that the depression of lymphocyte response to PHA in the presence of cancer serum is a non-specific effect of non-self serum. Depression of lymphocyte PHA response in cancer patients may be due to a primary or secondary lymphocyte defect.

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REFERENCES

1. D. B. Garrioch, R. A. Good and R. A. Gatti, Lymphocyte response to P.H.A. in patients with non-lymphoid tumours. *Lancet*, **i,** 618 (1970).
2. F. R. Eilber and D. L. Morton, Impaired immunologic reactivity and recurrence following cancer surgery. *Cancer* **25,** 362 (1970).
3. P. M. Bolton, S. L. James, J. M. Davidson and L. E. Hughes, Diagnostic and prognostic significance of delayed hypersensitivity skin testing in patients with malignant neoplasia. *Brit. J. Cancer* **28,** 80 (1973).
4. M. G. Whittaker, K. Rees and C. G. Clark, Reduced lymphocyte transformation in breast cancer. *Lancet* **i,** 892 (1971).
5. R. A. Gatti, Serum inhibitors of lymphocyte responses. *Lancet* **i,** 1351 (1971).
6. W. F. Sample, H. R. Gertner and P. B. Chretien, Inhibition of phytohaemagglutinin-induced *in vitro* lymphocyte transformation by serum from patients with carcinoma. *J. nat. Cancer Inst.* **46,** 1291 (1971).
7. P. J. Guillou, T. G. Brennan and G. R. Giles, Phytohaemagglutinin-stimulated transformation of peripheral and lymph-node lymphocytes in patients with gastro-intestinal cancer. *Brit. J. Surg.* **60,** 745 (1973).
8. N. Suciu-Foca, J. Buda, J. McManus, T. Thiem and K. Reemtsma, Impaired responsiveness of lymphocytes and serum-inhibitory factors in patients with cancer. *Cancer Res.* **33,** 2373 (1973).
9. H. S. Nelson, Delayed hypersensitivity in cancer patients: cutaneous and *in vitro* lymphocyte response to specific antigens. *J. nat. Cancer Inst.* **42,** 765 (1969).
10. E. K. Golob, T. Israsena, A. C. Quatrale and K. L. Becker, Effect of serum from cancer patients on homologous lymphocyte cultures. *Cancer* **23,** 306 (1969).
11. M. Al-Sarraf, S. Sardesai and V. K. Vaitkevicius, Effect of syngeneic and allogeneic plasma on lymphocytes from cancer patients, patients with non-neoplastic diseases and normal subjects. *Cancer* **27,** 1426 (1971).
12. M. B. McIllmurray, M. Gray and M. J. S. Langman, Effect of plasma from patients with large-bowel cancer on normal-lymphocyte responsiveness to P.H.A. *Lancet* **ii,** 452 (1973).
13. S. R. Cooperband, R. C. Davis, K. Schmid and J. A. Mannick, Competitive blockade of lymphocyte stimulation by a serum immunoregulatory alpha globulin (I.R.A.). *Transplant. Proc.* **1,** 517 (1969).
14. A. Bøyum, Separation of white blood cells. *Nature* (*Lond.*) **204,** 793 (1964).

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Factors Determining Cell Sensitivity to Methotrexate: Studies of Folate and Deoxyribonucleoside Triphosphate Pools in Five Mammalian Cell Lines

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Abstract—*Values are reported for cellular pools of folate cofactors and deoxyribonucleoside triphosphates in five cultured mammalian cell lines differing widely in their intrinsic resistance to methotrexate. No significant correlation was observed between cellular levels of folate or thymidine triphosphate and intrinsic drug sensitivity. A study has been made of the reversal of methotrexate toxicity by nucleosides. In some cell lines, partial rescue was achieved with thymidine alone, while in others both thymidine and deoxyadenosine were required. The effects of methotrexate and nucleosides on the deoxyribonucleoside triphosphate pools are reported and biochemical factors involved in the reversal of methotrexate toxicity by nucleosides are discussed.*

INTRODUCTION

MAMMALIAN cells vary greatly in their intrinsic sensitivity to the antifolate drug methotrexate (MTX). The toxic effects of this drug are thought to be a consequence of inhibition of dihydrofolate reductase [1, 2] and many studies of acquired resistance to MTX have shown increased cellular levels of this enzyme [3, 4]. There have been few studies of *intrinsic* MTX resistance and in general these have shown no correlation between cellular dihydrofolate reductase level and resistance to MTX [5, 6]. Impaired cellular permeability has also been implicated in acquired resistance to MTX [7, 8], but to our knowledge no correlation has been established between methotrexate transport and intrinsic sensitivity. Previous results from this laboratory indicated that in three of the cell lines used in the present study (Yoshida, L1210, L5178Y) neither methotrexate transport nor cellular levels of dihydrofolate reductase correlated with intrinsic resistance to methotrexate [9]. Consequently, it is necessary to consider what other factors might determine cellular sensitivity to this drug.

Reduced folate cofactors are required for the transfer of one-carbon units in both thymidylate and purine biosynthesis. MTX, by inhibiting dihydrofolate reduction, impairs the biosynthesis of thymidylate, leading to diminished cellular levels of thymidine triphosphate, TTP [10, 11]. Thus, one factor influencing sensitivity to MTX might be the cellular pools of reduced folates and a second, the cellular levels of TTP. Not only may intrinsic sensitivity to MTX vary by as much as 100-fold in different cell lines [5], but also experiments indicate that the nature of the disturbance of folate metabolism caused by the drug can show wide variation. For example, some cells are rescued from MTX toxicity if

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excess thymidine (Tdr) is present in their culture medium; others require purines as well as thymidine for complete rescue; yet other cells are most susceptible to the toxic effects of MTX in the presence of excess purines [12]. Clearly, the pattern of methotrexate toxicity in different cell lines shows wide variation and may depend on a number of factors of which the cellular deoxyribonucleoside triphosphate (dNTP) concentration and folate cofactor levels have been selected for study. Since the cofactor, 5,10-methylenetetrahydrofolate stands at the branch point between the thymidylate and purine biosynthetic pathways, it was also of interest to measure the relative activities of 5,10-methylenetetrahydrofolate dehydrogenase (EC.1.5.1.5) and thymidylate synthetase (EC. 2.1.1.b). Our results indicate that measurement of the dNTP pools and of the relative activities of these enzymes may be valuable in predicting the patterns of cellular response to methotrexate.

MATERIAL AND METHODS

Reagent chemicals were obtained from Hopkin and Williams Ltd. Nucleosides and nucleotides were purchased from Boerhinger and ^{3}H-dATP (14 Ci/mmol) and ^{3}H-TTP (23·7 Ci/mmol) from the Radiochemical Centre Amersham. Fischer's medium for leukaemic cells of mice was supplied by Grand Island Biological Company, New York and R.P.M.I. 1640 and Dulbecco's media from Bio-Cult Laboratories, Glasgow. Methotrexate was purchased from Lederle, and tetrahydrofolic acid from Sigma Chemicals. Poly-dAT was supplied by Boerhinger, calf thymus DNA by Sigma Chemicals and *Micrococcus luteus* DNA polymerase by Miles Laboratores.

Cell culture

Four of the cell lines studied have been maintained in suspension cultures for several years. The Yoshida sarcoma cells have been passaged in Chester Beatty Wistar rats for many years. These cells will grow in suspension culture for a few generations and primary cultures were studied in these experiments. The L5178Y mouse lymphoma cells and Yoshida cells were cultured in Fischer's medium, the W1-L2 human lymphoblastic cells [13] and L1210 mouse leukaemic cells in R.P.M.I. 1649, and the L cells (mouse fibroblasts) in Dulbecco's medium. Each medium was supplemented with 10% foetal calf serum, and in these conditions, the cells had the following doubling times: L1210—12 hr: L5178Y—12 hr; L cells—15 hr; W1-L2—19 hr; and Yoshida —22 hr. In our hands, dialysis of the foetal calf serum did not affect the response to methotrexate.

The sensitivity of the different cell lines to methotrexate, thymidine and deoxyadenosine (AdR) was measured by growth experiments with the drugs added to cultures which had been established 24 hr previously. Visual cell counts were performed daily in triplicate using a Fuchs Rosenthal haemocytometer.

Deoxyribonucleoside triphosphate extraction and assay

At the stated times, the cultures were placed in ice, and subsequently the cells collected by centrifugation at 350 **g** for 10 min in a refrigerated centrifuge. The cell pellet was washed in ice-cold PBS (phosphate-buffered saline) and recentrifuged. To the cell pellet, 1 ml of ice cold 60% methanol was added and the suspension stored at −20° for 16 hr. The precipitate was collected by centrifugation, hydrolysed in hot 0·5 M PCA (perchloric acid) and assayed colorimetrically for DNA [14]. The supernatant was evaporated to dryness in a rotary evaporator at 35°C. The dry extract was resuspended in 650 μl of water and stored at −20°C until assayed. The assay for dNTP has been described [10].

Enzyme assays

Cells were centrifuged at 350 **g** for 10 min, washed in 0·15 M sodium chloride and recentrifuged. The cell pellet was resuspended in 0·05 M Tris–HCl buffer pH 7·5 and disrupted with an MSE sonicator for 20 seconds. Insoluble material was removed by centrifugation at 25,000 **g** for 20 min.

The activity of 5,10-methylenetetrahydrofolate dehydrogenase was determined by the method of Scrimgeour and Huennekens [15], thymidylate synthetase was assayed by the spectrophotometric method of Dunlap *et al.* [16] using a Cary model 16K spectrophotometer. Measurements were carried out at 30°.

Folate extraction and assay

Extracts for the determination of folate cofactor pools were prepared as follows: cells were grown for 2 transfer generations in the absence of antibiotics. Cultures containing approximately 2×10^8 cells were centrifuged (350 **g** 20 min) and the pellet washed once in PBS. The washed cells were resuspended in 2·5 ml of 0·05 M phosphate buffer pH 6·0 containing 1% ascorbic acid. The lysate was placed in a boiling water bath for 15 min and

finally centrifuged (2000 **g** 20 min). Half of the resulting extract was incubated with a chicken pancreas conjugase preparation [17] for 12 hr at 37°C. Extracts, both conjugase treated and untreated, were assayed for folate cofactor content using *Lactobacillus casei* ATCC 7469, and *Streptococcus faecium* ATCC 8043; each sample was assayed at 5 concentrations with triplicate tubes at each concentration. Folic acid was used as standard [17].

RESULTS

Figure 1 shows MTX dose–response curves, plotted on a log–log basis, for the five cell lines.

Response in this context, refers to the reduction in cell count, relative to untreated controls, 48 hr after drug addition. The most resistant line (Yoshida cells) had an ID_{50} about 70 times that for L1210 (the most sensitive cell line studied). ID_{50} is defined as the drug concentration which reduces the cell number, 48 hr after drug addition, to 50 % of the value for untreated controls.

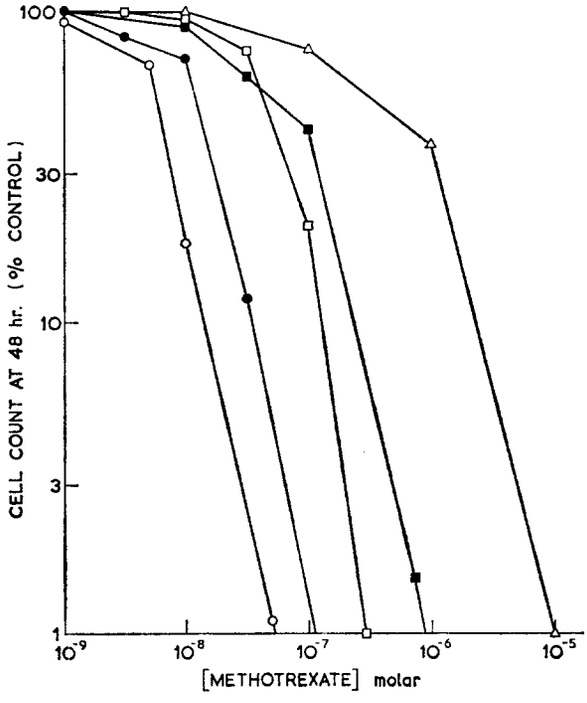


Fig. 1. *Dose–response curves of cultured cell lines to methotrexate. Cells were set up at a concentration of* 5×10^4*/ml in the appropriate medium. After* 48 *hr, cell counts were compared with those of untreated controls. All points represent means of triplicate cultures.*

—○— *L1210;*
—●— *W1-L2;*
—□— *L cells;*
—■— *L5178Y;*
—△— *Yoshida.*

ID_{50} values(as defined in Results section) were as follows: L1210 $5{\cdot}8 \times 10^{-9}M$; *W1-L2* $1{\cdot}3 \times 10^{-8}M$; *L cells* $4{\cdot}4 \times 10^{-8}M$; *L5178Y* $5{\cdot}6 \times 10^{-8}M$; *Yoshida* $3{\cdot}9 \times 10^{-7}M$.

Neither thymidine nor deoxyadenosine administered separately or simultaneously, influenced the cellular growth rate in any of the cell lines studied; Fig. 2 shows typical results, those obtained with L5178Y.

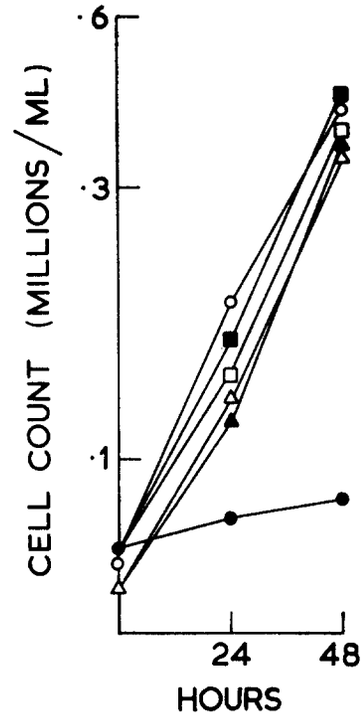


Fig. 2. *Effects of nucleosides on growth,* in vitro, *of L5178Y cells ± methotrexate.*

—○— *Controls;*
—●— *MTX* $10^{-6}M$
—□— *MTX* $10^{-6}M$ + *AdR,* $4 \times 10^{-5}M$ + *TdR* $4 \times 10^{-5}M$
—▲— *TdR* $4 \times 10^{-5}M$
—△— *AdR* $4 \times 10^{-5}M$
—■— *AdR* $4 \times 10^{-5}M$ + *TdR* $4 \times 10^{-5}M$

Experiments with methotrexate used concentrations of the drug which were lethal for each cell line under study; for L1210 and W1-L2 the methotrexate concentration in all experiments was 10^{-7}M, for L cells it was 5×10^{-7}M, for L5178Y 10^{-6}M, and for the Yoshida sarcoma it was 10^{-5}M. In all these cell lines, complete reversal of the toxicity of the indicated concentration of drug was obtained by addition to the culture medium, at the same time as methotrexate, of thymidine plus AdR, both to a concentration of 4×10^{-5}M (Fig. 2.).

Some of the cell lines studied could be partially rescued from methotrexate toxicity if thymidine (4×10^{-5}M) were present in the culture medium. Figure 3 indicates that the ability of thymidine to reverse methotrexate toxicity showed wide variation among the cell lines studied. In the W1-L2 and Yoshida

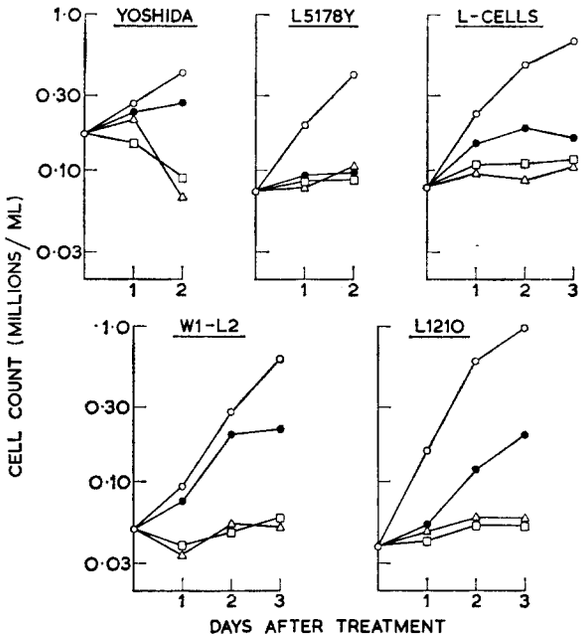


Fig. 3. Reversal of MTX toxicity by thymidine and deoxyadenosine. Growth conditions were as described in the methods section. MTX concentrations used were: Yoshida $10^{-5}M$; L51787 $10^{-6}M$; L-cells $5 \times 10^{-7}M$; W1-L2 and L1210 $10^{-7}M$. TdR and AdR concentrations were both $10^{-5}M$.

—○— *Controls*
—□— *+ MTX*
—●— *MTX+TdR*
—△— *MTX+AdR.*

cultures, thymidine addition resulted in an appreciable degree of rescue. In L1210 cells, the degree of rescue was less, but the cell number was continuing to increase after 72 hr. In L-cells the rescue was even less effective, and only temporary, whilst in L5178Y cultures, thymidine addition had virtually no effect on methotrexate toxicity. Deoxyadenosine alone (4×10^{-5}M) did not rescue any of the cell lines; in the L-cells and W1-L2 cells, the presence of deoxyadenosine even slightly enhanced the cytotoxicity of the methotrexate.

The cellular levels of folate cofactors are summarized in Table 1. The highest total folate levels were found in the Yoshida and the L1210 cells, and the lowest levels in L-cells. Neither the total folate nor any of the subfractions studied showed any clear correlation with the cellular sensitivity to methotrexate. Also shown in Table 1 are folate cofactor concentrations in a number of L1210 and L5178Y mutant sublines with *acquired* MTX resistance. In no case was the value appreciably different from that of the parent line. The ability of thymidine alone or thymidine in combination with purine to rescue cells from MTX toxicity will depend upon partition of cellular tetrahydrofolate cofactors between thymidylate biosynthesis on the one hand, and purine nucleotide biosynthesis on the other. This will be determined in part by the relative activities of thymidylate synthetase and 5,10-methylenetetrahydrofolate dehydrogenase. Consequently, we have measured activities of both enzymes in the five cell lines (Table 2): the significance of these results is discussed below.

Since methotrexate influences the biosynthesis both of thymidine and purine nucleotides, we have measured cellular pools of deoxyribonucleoside triphosphates, the immediate precursors of DNA (Table 3). In all cell lines TTP is the most abundant deoxyribonucleoside triphosphate. Whereas there appears to be

Table 1. Cellular levels of folate cofactors

	L. Casei microbioassay		*S. faecium* microbioassay	
	Before conjugase	After conjugase	Before conjugase	After conjugase
	n-moles/10^9 cells		n-moles/10^9 cells	
Yoshida	16.6 (2)	35·7 (2)	10·4 (2)	22·7 (2)
L5178Y	6·7 (3)	15·2 (3)	4·0 (3)	8·8 (3)
L5178Y/R1	6·3 (2)	14·2 (2)	3·2 (2)	7·2 (2)
L-cells	4·0 (2)	8·7 (2)	2·9 (2)	6·0 (2)
L1210	8·4 (5)	23·7 (5)	7·6 (3)	12·9 (3)
L1210/R2	9·6 (2)	26·2 (2)	—	—
L1210/R3U	7·8 (2)	24·6 (2)	7·3 (2)	10·7 (2)
L1210/R6	8·1 (2)	30·0 (2)	—	—
W1-L2	8·9 (2)	23·2 (2)	7·0 (2)	13·1 (2)

Figures in parentheses are number of cultures assayed.
Levels in each culture were determined using triplicate tubes at each of 5 concentrations, folate being used as standard. See Methods section for assay details. L1210R2 is an eight-fold methotrexate-resistant subline containing elevated dihydrofolate reductase; L1210/R3U is a 10-fold resistant subline with defective methotrexate transport; L1210/R6 is 80-fold resistant, and contains elevated dihydrofolate reductase. L5178Y/R1 is a 10-fold resistant mutant with defective methotrexate transport. A hyphen indicates that no value is available for the point in question.

some correlation between the TTP pool and intrinsic resistance to methotrexate, the differences are small and certainly insufficient to account for the observed variation in MTX sensitivity.

Table 2. *Thymidylate synthetase and* 5, 10-*methylene-tetrahydrofolate dehydrogenase activities in cultured cell lines*

	Thymidylate synthetase (T.S.) units	5, 10-methylene-tetrahydrofolate dehydrogenase (DH) units	Ratio: DH/T.S.
L1210	0·30	5·2	17·3
W1-L2	0·17	4·1	24·1
L-cells	0·23	3·7	16·1
L5178Y	0·45	5·0	11·1
Yoshida	0·12	4·5	39·5

Enzyme units are n-mole of substrate converted/min/mg protein.

Table 3. *Deoxyribonucleoside triphosphate pools*

Cell type	TTP *p*-mole/μg DNA	dATP *p*-mole/μg DNA	dCTP *p*-mole/μg DNA	dGTP *p*-mole/μg DNA
L1210	0·85	0·08	0·18	0·06
W1-L2	0·54	0·06	0·02	0·03
L-cells	0·86	0·19	0·08	0·07
L5178Y	1·3	0·11	0·23	0·09
Yoshida	2·6	0·13	0·23	0·06

Scatter $\pm$ $\pm$5% for TTP, dATP and dCTP and $\pm$ $\pm$20%, for dGTP.

Although deoxyadenosine and thymidine, each at a concentration of 4×10^{-5}M did not influence cellular gowth rate, they did have a marked effect on cellular pools of dNTP (Table 4). In all cell lines, thymidine addition, with or without deoxyadenosine, increased the TTP pool, and reduced the deoxycitidine triphosphate (dCTP) pool. Deoxyadenosine in presence of thymidine, led to a decrease in the deoxyadenosine triphosphate (dATP) pool. Methotrexate, at the lethal concentrations stated, caused a consistent reduction in the TTP pool of all five cell lines (Table 5). The dATP and dCTP pools were either relatively little changed or increased. Table 6 indicates that in each cell line, adminstration of thymidine at the same time as MTX prevented the fall in TTP level caused by MTX alone. Under these conditions, pools of dCTP, dATP, and deoxyguanosine triphosphate (dGTP) are reduced. The addition of deoxyadenosine and thymidine to cultures containing MTX prevents the expansion of the dATP pool caused by MTX alone.

Table 5. *dNTP pools following methotrexate treatment for* 24 *hr* (% *of control values*)

Cell type	TTP	dATP	dCTP	dGTP
L1210	29	290	220	95
W1-L2	50	300	70	34
L-cells	35	110	95	35
L5178Y	44	110	297	49
Yoshida	85	250	210	190

For methotrexate concentration in each cell line see caption to Fig. 3.

DISCUSSION

Methotrexate is used widely to treat a variety of human malignant diseases. Two major factors have limited its value, (a) the toxic effects of the drug are not confined to tumour cells but may be severe in normal cells and (b) tumour cells frequently become insensitive to repeated drug treatment. Since tumour cells vary greatly in their intrinsic sensitivity to methotrexate, an understanding of the biochemical basis for this variation might be helpful in selecting patients with drug sensitive neoplasms. Moreover, the inhibition of dihydrofolate reductase by methotrexate produces different consequences in different cells. It therefore might be possible to focus the toxic effects of methotrexate on tumour cells rather than normal cells if the biochemical

Table 4. *dNTP pools: effect of added deosyadenosine and thymidine* ($4 \times 10^{-5}M$) *at* 24 *hr* (% *of control values*)

	Thymidine				Thymidine and deoxyadenosine			
Cell type	TTP	dATP	dCTP	dGTP	TTP	dATP	dCTP	dGTP
L1210	120	18	80	170	120	1	85	150
W1-L2	138	200	34	32	180	2	60	0
L-cells	110	10	21	190	128	14	1	160
L5178Y	151	200	27	28	130	15	32	3
Yoshida	300	60	24	13	350	50	29	13

basis for these differences could be identified.

Two factors have been shown to be responsible for acquired methotrexate resistance in tumour cells: (a) raised total dihydrofolate reductase level [3, 4] and (b) impaired methotrexate transport [5, 8, 9]. These mechanisms are not the basis for the variation in intrinsic sensitivity to methotrexate in the five cultured cell lines studied here, (Ref. 5 and unpublished results of this laboratory) since dihydrofolate reductase activity and the methotrexate transport characteristics are similar in each of the cell lines. The possibility that large stores of reduced folate might enable cells to withstand a methotrexate challenge has been examined. The total folate pools in these cell lines vary but there is no correlation with sensitivity to methotrexate. Moreover, in resistant mutants the total folate pools do not differ from those in the parent lines. The microbiological assays used do not distinguish between folic acid and reduced folates. However, most of the folate found in mammalian cells consists of tetrahydrofolate forms with little or no folic acid or dihydrofolate [18] and thus it is justifiable to conclude that in these cells, stores of reduced folate do not correlate with methotrexate resistance. The possibility that increased pools of TTP might contribute to cellular resistance to MTX has also been examined. The TTP pools in the different cell lines do not correlate well with overall sensitivity to MTX, though Yoshida cells which are the least sensitive cells studied, have the largest TTP pool (Table 3). Since at the cellular growth rates studied, the measured pools of TTP would only sustain DNA biosynthesis for a few minutes, the small differences observed would only delay the response to MTX for a very short time.

The extent to which thymidine and deoxyadenosine will reverse methotrexate toxicity in the five different cell lines has been studied. In agreement with published data, L5178Y cells required the presence of thymidine and deoxyadenosine to be rescued from the effects of methotrexate [19]. The L-cells were rescued by thymidine only to a slight extent, but deoxyadenosine alone appeared to increase the cytotoxic effects of methotrexate (also the case for W1-L2 cells) and under these conditions unbalanced growth was most marked. These findings are in agreement with the results of Borsa and Whitmore, who showed that addition of deoxyadenosine increased the cytotoxicity of MTX as measured by cloning experiments [20]. Thymidine rescued the Yoshida, W1-L2 and L1210 cultures to an appreciable degree, and if deoxyadenosine were also present, all five cell lines were rescued completely (Fig. 1 and text).

The presence of excess deoxyadenosine and thymidine in the culture medium did not influence cell growth in the five cells but did disturb the deoxyribonucleoside triphosphate pools (Table 4). The most striking effect was an increase in the TTP pools in all cell lines with reduction in dCTP pools. No consistent effect on the purine deoxyribonucleoside pools was observed. It must be assumed that these changes are insufficient to interfere with cell growth.

Morris and Fischer [22] and Maley and Maley [21], have observed that thymidine toxicity is reversed by deoxycytidine, but not by cytidine. Our results show that expansion of TTP pools by thymidine is associated with depression of dCTP pools. It thus seems likely that the mechanism of thymidine toxicity is a limitation in the availability of dCTP.

Our findings, reported here, that MTX caused a general reduction in TTP pools confirms and extends our previous observations [10]. The TTP is present in at least five-fold excess over the concentrations of the other three dNTP's, and is reduced in the presence of MTX to about half the control values. It is

Table 6. Effect of excess thymidine ($4 \times 10^{-5} M$) *and deoxyadenosine* ($4 \times 10^{-5} M$) *on the deosyribonucleoside triphosphate pool changes caused by a 24 hr exposure to methotrexate*

	MTX + TdR				MTX + AdR + TdR			
Cell type	TTP	dATP	dCTP	dGTP	TTP	dATP	dCTP	dGTP
L1210	250	1	27	29	125	1	27	80
W1-L2	150	1	100	12	110	95	70	67
L-cells	130	7	22	23	80	10	50	15
L5178Y	155	2	17	7	110	5	13	25
Yoshida	370	25	10	4	400	17	25	25

For methotrexate concentration in each cell line see caption to Figure 3.

clear that this depression, while leading to cell death, still leaves TTP in considerable excess. The explanation for this situation is currently under investigation in these laboratories. In all cell lines, thymidine, administered concurrently with MTX prevented the drop in TTP levels caused by the drug. In fact, under these conditions, TTP pools were higher than the control values. Notwithstanding this, thymidine failed to rescue all cells from the toxic effects of MTX. Methotrexate produced either an expansion or a modest decrease in the purine dNTP pools (Table 5). However, in striking contrast to these results, the concurrent administration of methotrexate and thymidine produced a pronounced and consistent fall in the purine dNTP pools (Table 6). It is clear that in the presence of excess thymidine, methotrexate exerts a predominantly anti-purine effect.

The extent to which cells require thymidine, or both thymidine and deoxyadenosine for reversal of MTX toxicity must be a reflection of the way in which the 5,10-methylenetetrahydrofolate is compartmented between the various pathways using this co-factor. The ratio of the relative activities of 5,10-methylenetetrahydrofolate dehydrogenase and thymidylate synthetase in the 5 cell lines show some correlation with the degree of thymidine reversal of methotrexate toxicity (Table 2). The value of this ratio is highest in those cell lines (Yoshida W1-L2, L1210) which are most successfully rescued by thymidine. The value of this ratio is no doubt attributable to the association of thymidylate synthetase with S phase of the cell cycle, [23] whereas the dehydrogenase is not specifically associated with S phase but is present throughout the cell cycle. Thus, in an asychronous cell population, those cells with a long generation time will have low mean levels of thymidylate synthetase and a high ratio of dehydrogenase to synthetase activity. This finding may, therefore, allow for prediction of this response in other tumour cells. Clearly the critical factor is not solely the relative V_{max} of the two enzymes (as measured here) but also the extent of saturation with cofactor. At present there are not sufficient kinetic data available to make this comparison. Similarly the total folate pools correlate, not with drug sensitivity *per se* but with the ability of thymidine to reverse methotrexate toxicity. The possible explanation of these two findings is clear if one considers the interrelationship of the folate cofactors required for purine and thymidylate biosynthesis. If 5,10-methylenetetrahydrofolate dehydrogenase is relatively active, folate cofactors will be concentrated in those forms required for purine biosynthesis, and thus excess thymidine will, to a large extent, balance thymidylate and purine biosynthesis. Similarly, where tetrahydrofolate pools are large, purine biosynthesis will be sustained longer in the presence of MTX and therefore, thymidine will be more likely to rescue treated cells.

Although the present study has cast little light on the pattern of intrinsic resistance to antifolate, some factors which influence the differential responses of MTX-treated cells to nucleosides have been identified. It is recognised that these parameters are only a small part of the complex control of folate metabolism; nevertheless differential rescue experiments may provide a means of optimising antifolate chemotherapy. For example, if normal bone marrow cells require only excess thymidine to be rescued from MTX toxicity but tumour cells require both purines and thymidine, it might be possible, by artificially modifying the plasma levels of these nucleosides, to obtain more selective antitumour effects than could be achieved using this drug conventionally. It remains to investigate the factors governing the "rescueability" of normal proliferating tissues from antifolate toxicity. A knowledge of the normal plasma concentrations of nucleosides may be of critical importance in this respect.

REFERENCES

1. S. Futterman and M. Silverman, The "inactivation" of folic acid by liver. *J. biol. Chem.* **224,** 31 (1957).
2. M. J. Osborn, M. Freeman and F. M. Huennekens, Inhibition of dihydrofolic reductase by aminopterin and amethopterin. *Proc. Soc. exp. Biol.* **97,** 429 (1958).
3. G. A. Fischer, Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukaemic cells. *Biochem. Pharmacol.* **7,** 75 (1961).
4. U. D. K. Misra, S. R. Humphreys, M. Freidkin, A. Goldin and E. J. Crawford, Increased dihydrofolate reductase activity as a possible basis of drug resistance in leukaemia. *Nature (Lond.)* **189,** 39 (1961).

5. D. Roberts, I. Wodinsky and T. C. Hall, The level of enzyme activity and response to methotrexate of transplantable mouse tumours. *Cancer Res.* **25,** 1899 (1965).
6. D. Roberts and I. Wodinsky, On the poor correlation between the inhibition by methotrexate of dihydrofolate reductase and of deoxyribonucleoside incorporation into DNA. *Cancer Res.* **28,** 1955 (1968).
7. G. A. Fischer, Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y Leukaemic Cells. *Biochem. Pharmacol.* **11,** 1233 (1962).
8. F. M. Sirotnak, S. Kurita and D. J. Hutchison, On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukaemia. *Cancer Res.* **28,** 75 (1968).
9. K. R. Harrap, B. T. Hill, M. E. Furness and L. I. Hart, Sites of action of amethopterin: intrinsic and acquired drug resistance. *Ann. N.Y. Acad. Sci.* **186,** 312 (1971).
10. M. H. N. Tattersall and K. R. Harrap, Changes in the deoxyribonucleoside triphosphate pools of mouse 5178Y lymphoma cells following exposure to methotrexate or 5-fluorouracil. *Cancer Res.* **33,** 3086 (1973).
11. A. V. Hoffbrand and E. Tripp, Unbalanced deoxyribonucleotide synthesis caused by methotrexate. *Brit. Med. J.* **2,** 140 (1972).
12. J. Borsa, Cytotoxic mechanisms of folate antagonists. *Ann. N.Y. Acad. Sci.* **186,** 359 (1971).
13. J. A. Levy, M. Virolainen and V. Defendi, Human lymphoblastoid lines from lymph node and spleen. *Cancer* **22,** 517 (1968).
14. 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).
15. C. K. Matthews, K. G. Scrimgeour and F. M. Huennekens, Dihydrofolic Reductase. In *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. VI, p. 364. Academic Press, London (1963).
16. R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei. Biochemistry* **10,** 88 (1971).
17. O. D. Bird, V. M. McGlohon and J. W. Vaitkus, Naturally occurring folates in the blood and urine of the rat. *Analyt. Biochem.* **12,** 18 (1965).
18. M. Silverman, J. C. Keresztesy, G. J. Koval, and R. C. Gardiner, Citrovorum factor and the synthesis of formyl glutamic acid. *J. biol. Chem.* **226,** 83 (1957).
19. W. M. Hryniuk, Purineless death as a link between growth rate and cytotoxicity by methotrexate. *Cancer Res.* **32,** 1506 (1972).
20. J. Borsa and G. F. Whitmore, Cell killing studies on the mode of action of methotrexate on L-cells *in vitro. Cancer Res.* **29,** 737 (1969).
21. F. Maley and G. F. Maley, On the nature of a sparing effect by thymidine on the utilisation of deoxycytidine. *Biochemistry* **1,** 847 (1962).
22. N. R. Morris and G. A. Fischer, Studies concerning inhibition of the synthesis of deoxycytidine by phosphorylated derivatives of thymidine. *Biochim. Biophys. Acta (Amst.)* **42,** 183 (1960).
23. F. Maley and G. F. Maley, Elevation of deoxycytidine deaminase and thymidylate synthetase in regenerating rat liver. *J. biol. Chem.* **235,** 2968 (1960).

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The Haematopoietic Stem Cell in Rauscher Virus-induced Erythroblastosis of BALB/c Mice

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Abstract—*The relative number of haematopoietic stem cells in the spleens of Rauscher erythroblastosis-virus infected BALB/c mice remains approximately constant throughout the course of the disease. These stem cells have a normal differentiative capacity as judged from the distribution of histological types of spleen colonies in irradiated recipients. Incubation of spleen cell suspensions with rabbit antisera to the virus leads to a drastic reduction in stem cells, indicating the presence of the virus in these cells. A lesser sensitivity to normal rabbit serum indicates the partial loss of xenogeneic antigens on the surface of infected stem cells.*

INTRODUCTION

KNOWLEDGE of the role of the haematopoietic stem cell in leukaemia is of more than academic interest. Present day therapeutic regimens are based on the assumption that a normal stem cell pool will take over the production of haematopoietic cells after eradication of the leukaemic bone marrow by cytotoxic agents. Relapse of leukaemia is explained by the persistence of one or a few leukaemic stem cells or by the leukaemic alteration of previously normal precursor cells as, e.g., after bone marrow transplantation [1]. An alternative to these hypotheses has been provided by Killmann [2] in the "leukaemia major-leukaemia minor" theory in which all haematopoietic stem cells might be affected by the disease but may give rise to a seemingly normal progeny under optimal conditions.

In animal leukaemias, among which those induced by Rauscher and Friend murine leukaemia viruses are the most extensively studied, the ideas concerning the role of the stem cell are divergent. This study was an attempt to resolve the problem with respect to Rauscher virus-induced leukaemia of BALB/c mice.

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MATERIAL AND METHODS

Female BALB/c, 4 weeks of age, were injected intraperitoneally with 0·2 ml of a 10% cell-free extract of enlarged spleens of mice infected with Rauscher leukaemia virus (RLV). The virus preparation was stored in liquid nitrogen. The virus was originally obtained from the Netherlands Cancer Institute, Amsterdam, to which it was provided in 1967 by Dr. F. Rauscher Jr., National Cancer Institute, Bethesda, Maryland (USA). Upon passage in the Radiobiological Institute TNO, the virus changed its titration pattern in the spleen focus assay [3]. The latter preparation has been used in this investigation.

Spleen colony assays were performed according to the method of Till and McCulloch [4]. Spleen cell suspensions were prepared in Hanks' balanced salt solution (HBSS). Viable

Table 1. Number of colony forming units per 10^6 spleen cells of BALB/c mice infected with RLV, determined at various intervals after the infection. Between brackets are the numbers of spleens in which the colonies were counted

Days after RLV-inoculation of donors	Experiment Ia (pooled spleens)		Experiment Ib (single spleens)			Experiment II (pooled spleens)
0	75 [13]	100 [18]	—	31 [8]	29 [8]	—
1	—	—	—	—	—	93 [15]
3–4	60 [9]	29 [15]	47 [12]	39 [17]	27 [8]	99 [18]
7–8	66 [14]	—	—	—	—	—
10–11	—	25 [8]	27 [11]	55 [17]	34 [13]	103 [15]
14–15	86 [13]	—	—	—	—	61 [15]
17–18	—	—	31 [13]	25 [29]	—	—
21–22	15 [12]	—	39 [8]	22 [4]	—	109 [20]
28–29	39 [20]	—	—	—	—	—

cells were counted in trypan blue and the original suspension was diluted with HBSS to different concentrations ranging from 2×10^5 to 2×10^6 cells per ml. Aliquots of 0·5 ml of these suspensions were injected intravenously into syngeneic mice under light ether anaesthesia. The recipients (10-weeks-old female BALB/c mice preirradiated with 700 rads whole body irradiation 1–4 h prior to the injection of spleen cells) were sacrificed 10 days later. The spleens were fixed in Tellyesniczky's solution. Superficial colonies were counted with the naked eye. The number of colonies produced by 10^6 cells was calculated for each dilution of cells injected. The mean of these figures was referred to as the number of colony forming units (CFU). Background counts in simultaneously irradiated control mice were negligible; less than one count in 20 spleens.

For the histological evaluation of the cell types within the colonies, the spleens were fixed in Bouin's solution, processed in an Autotechnicon 2A (Technicon Company, Chauncey, New York, USA), embedded in paraffin, sectioned semiserially, and stained with haematoxilin–phloxin–saffran.

Antiserum against RLV was collected from rabbits which had been injected with virus isolated from plasma of leukaemic mice [5] together with complete Freund's adjuvant. The antiserum was absorbed by mixing equal volumes of rabbit serum and a normal mouse spleen cell suspension containing 10^7 cells per ml and 1 mg dry frozen normal mouse serum, and incubating for 45 min at 37°C. This mixture was repeatedly resuspended by gentle shaking. After incubation, the serum was separated by centrifugation for 15 min at 2000 rev/min, collected, and stored at −20°C until use. The antiserum was tested for reactivity with the virus by (a) Ouchterlony immunodiffusion; (b) indirect immunofluorescence of infected and noninfected spleen cells; and (c) neutralization of the virus as estimated by inhibition of splenomegaly 3 weeks post inoculation of the reaction-mixture.

To evaluate the effect of antiserum upon the colony forming ability of the spleen cell suspensions, these suspensions (10^7 cells per ml) were incubated with antiserum for 45 min at 37°C. The number of cells injected into mice was based on cell counts made before the incubation with the antiserum. Spleen colony assays were performed as described previously.

RESULTS

All mice which received a high dose of RLV i.p. at the age of 4 weeks responded with rapid growth of the spleen, deterioration of the general condition, and death within 3 or 4 weeks. Immature erythroid cells accumulated in the spleen, the liver, and eventually in the bone marrow as described by others [6, 7]. The animals were anaemic and thrombocytopenic [8]. The leukocyte count was considerably elevated. This was mainly due to granulocytosis which was accompanied by either lymphocytosis or erythroblastosis [7, 9].

The number of colony-forming units in the spleen cell suspensions was measured at various intervals after the infection with RLV. Two different procedures were followed. In Experiment I, donor mice were injected simultaneously and, thereafter, a group of four mice was sacrificed twice a week; spleen cell suspensions were prepared from either the four spleens pooled together (Ia) or from single spleens (Ib). In Experiment II, groups of donor mice were infected at different times before sacrifice. One day after the infection of the last group, all mice of this experiment were killed on the same day. The colony assays for all groups could thus be performed simultaneously.

The results of these experiments are shown in Table 1 and Fig. 1. The number of CFU per 10^6 spleen cells remained approximately constant in spite of the massive enlargement of the spleen during the same period of time. The addition of RLV to normal spleen cell suspensions did not have any influence on the number of colonies in the irradiated recipients (Table 2).

The results of the analysis of one hundred colonies produced by normal spleen cells were compared with those of the same number of colonies produced by spleen cells of leukaemic mice 3 weeks after infection (Table 3). Most colonies contained more than one cell type. Some consisted of primitive cells, the origin of which could not be determined. The only difference between the two groups was a slightly greater number of erythroid colonies produced by leukaemic spleen cells. From this experiment, it may be concluded that the colony-forming cells of the erythroblastic spleens retain their pluripotency.

The survival of lethally irradiated mice

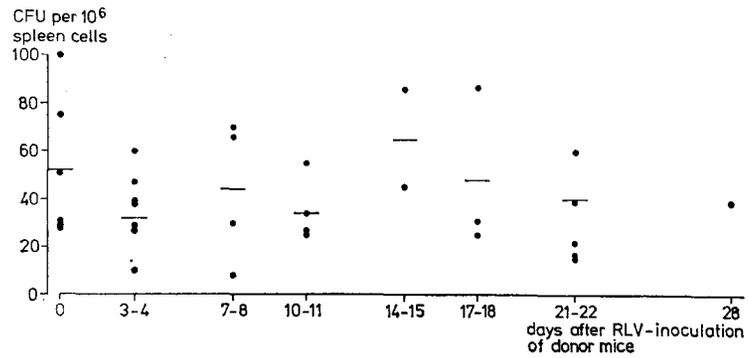


Fig. 1. *Colony forming units (CFU) per* 10^6 *spleen cells obtained from BALB/c mice at various intervals after infection with RLV. The data are from three different experiments.*

Table 2. *Spleen colony assay of normal spleen cell suspensions with and without the addition of RLV* in vitro. *The results of two separate experiments* (a) *and* (b) *are reported;* N = *number of spleens in which colonies have been counted*

Number of cells injected with and without RLV	*N*	Number of colonies counted per spleen (mean)	CFU per 10^6 injected spleen cells	
(a) 2×10^5	3	2·0	10·0	7·7
(b) 2×10^5	10	1·1	5·5	
(a) 5×10^5	6	6·1	12·2	8·3
(b) 5×10^5	9	2·2	4·4	
(a) 2×10^5 + RLV	1	1·0	5·0	8·0
(b) 2×10^5 + RLV	5	2·2	11·0	
(a) 5×10^5 + RLV	3	3·6	7·2	8·1
(b) 5×10^5 + RLV	4	4·5	9·0	

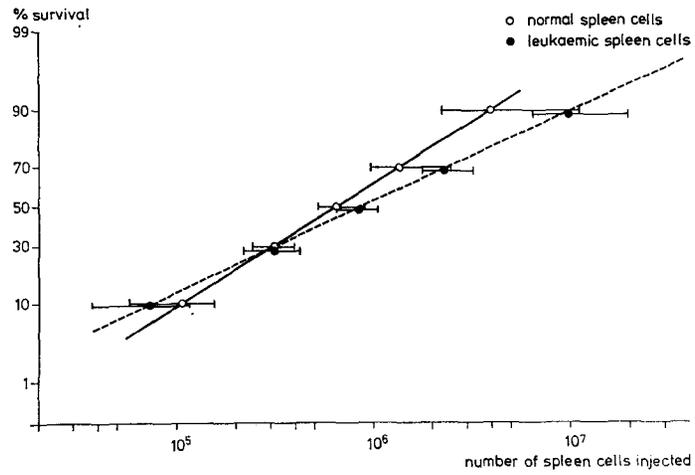


Fig. 2. Probit curves of the survival of BALB/c mice 30 days after lethal irradiation and restoration with leukaemic spleen cells vs normal spleen cells; 95% confidence limits indicated by horizontal bars.

injected with leukaemic spleen cells was compared with the survival of similarly treated mice infected with normal spleen cells. The results of several identical experiments were collected and conformed to a probit curve (Fig. 2). There is no significant difference in the number of cells necessary for the survival of 50% of the lethally irradiated mice for at least 30 days. It may be concluded that the repopulating capacity of the stem cells remains normal in the erythroblastic spleens.

Table 3. Spleen colonies in lethally irradiated mice, 10 days after the injection of normal (N) and leukaemic (L) spleen cells, arranged according to the type of cells constituting the colony. Between brackets are the numbers of colonies in each category consisting apparently of only one cell type

	Erythroid	Myeloid	Mega-karyotic	Undeter-mined	Total
N	66 [20]	68 [20]	22 [3]	9 [4]	100
L	71 [35]	51 [11]	24 [5]	9 [5]	100

Table 4. Cytotoxic effects of rabbit anti-RLV antiserum on the colony forming cells of normal and leukaemic spleens, compared to the effect of suspension medium, non-absorbed and absorbed normal rabbit serum. Each figure represents the mean colony count in 20 spleens 10 days after grafting of the incubated cells in lethally irradiated recipients

Incubation medium	CFU/10^6 spleen cells Normal spleen cells	Leukaemic spleen cells
Hank's balanced salt solution	28	130
Normal rabbit serum	1	40
Normal rabbit serum—abs NMSC*	38	155
Anti-RLV I	10	2
Anti-RLV I—abs NMSC*	30	4
Anti-RLV II	9	18
Anti-RLV II—abs NMSC*	36	41

*abs NMSC = absorbed with normal mouse spleen cells.

Rabbit antisera against RLV almost completely abolished the spleen colony forming ability of the CFU from erythroblastic spleens (Table 4). This effect was not influenced by absorbing the antisera with normal mouse spleen cells, which confirmed the specificity of the antibodies against RLV. Curiously, the incubation of the leukaemic cell suspensions with normal rabbit serum did not change the number of CFU. This was in contrast to normal mouse spleen cells, in which the number of CFU decreased dramatically following the same treatment.

DISCUSSION

The most important manifestation of Rauscher leukaemia is erythroblastic proliferation which results in splenomegaly, hepatomegaly, anaemia, thrombocytopenia, and impairment of the general condition. Many investigations have been undertaken with the intention of determining the target of the virus. Indications have been obtained that an erythroblastic precursor cell may play such a role [10]. However, most data can also be explained by the assumption that the virus primarily attacks the pluripotent stem cell and that secondary factors influence the outcome of the disease.

The number of CFU per 10^6 spleen cells remains rather constant throughout the course of the infection, even in mice with an enormous splenomegaly and a high count of nucleated

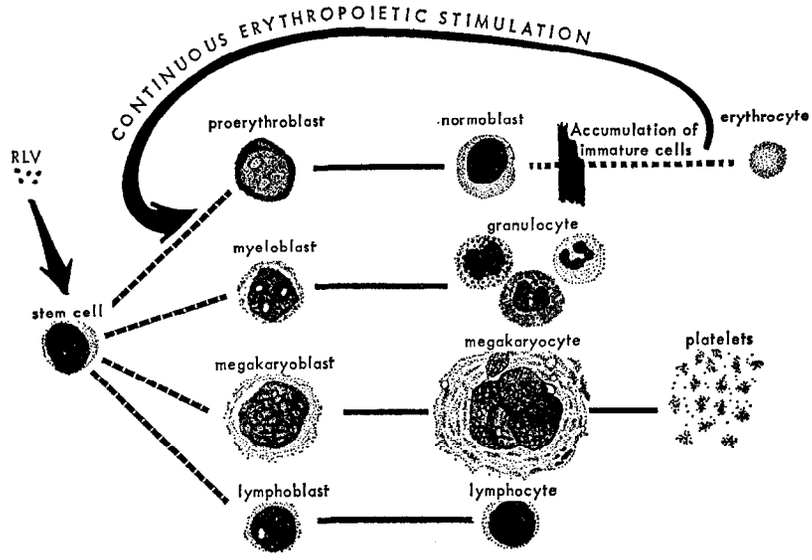


Fig. 3. *Schematic representation of the development of RLV-induced erythroblastosis.*

cells in the peripheral blood. This implies that the total number of colony forming units per spleen must have increased by the same order of magnitude as the total spleen cell number, i.e. 20- to 30-fold. Comparable increases in the number of CFU per spleen have been observed for RLV in other mouse strains [11, 12] and for other erythroblastosis-inducing viruses [13–15]. The relatively high number of colonies cannot be due to the presence of virus in the spleen cell inoculum since injection of cell-free virus preparations does not result in colony formation in lethally irradiated recipients [16] and in our experience, admixture of virus with the spleen cell suspensions *in vitro* did not influence the number of colonies. The histological analysis of the spleen colonies indicates the normal differentiating capacity of the infected stem cells.

The observed constancy of the number of CFU per 10^6 cells is in accord with the results of the second experiment in which the repopulating capacity of leukaemic spleen cells was measured in lethally irradiated mice. The bone marrow syndrome resulting from the irradiation could be prevented by the transplantation of leukaemic cells as effectively as with normal spleen cells. It follows that the leukaemic stem cells retain their repopulating capacity and do not differ from normal spleen cells in this respect. However, the thrombocyte counts remained considerably low, approx. 10% of normal, as also observed in similar experiments with the polycythemia-inducing Friend virus [17].

This observation does not exclude functional integrity of the total number of spleen stem cells in Rauscher disease. The fact that the proliferation of CFU seems to occur at the same rate as cells suggests physiological regulation rather than leukaemic proliferation; this would be in accord with this presumed functional integrity of the stem cells.

Evidence has been obtained for the presence of Rauscher virus in stem cells of leukaemic spleens: specific antisera against RLV abolished spleen colony formation by leukaemic cell suspensions but not of normal cells. This effect must be attributed to the presence of viral antigens on the surface of colony forming cells. Obviously, the presence of the virus in the stem cells is not prohibitive for colony formation and maturation of functioning end cells (repopulation of radiation damaged bone marrow). The lack of cytotoxic activity of normal rabbit serum to infected stem cells indicates the reduction in xenogeneic antigens on the cell surface, as recently described for Rous sarcoma virus-transformed hamster cells [18].

In the light of the presented experimental data, it does not seem valid to maintain the concept of a specific target cell for Rauscher virus: among other cells, the virus invades different types of haematopoietic cells, the stem cell included. The fact that only a certain cell line is involved in leukaemia induced by a given virus cannot be the consequence of the replication of the virus in the proliferating cell line but must be due to a more subtle mechanism such as the interference of the viral genome with the genome of differentiating cells or with substances which regulate differentiation. In the case of Rauscher virus-induced erythroblastosis either exogenous erythropoietin or stimulation of endogenous erythropoietin production by

hypoxia intensifies the erythroblastic response, whereas suppression of erythropoiesis by polycythaemia diminishes the effect of the virus [10, 19, 20]. These observations were considered originally as evidence for the concept of a primitive erythroblast being the target for the virus. We propose the alternative hypothesis that stimulation of infected stem cells to differentiate into the erythroid direction leads to the leukaemic alteration, whereas differentiation into either one of the other three haematopoietic lines can proceed in a relatively normal fashion (Fig. 3). In contrast to Friend virus-induced erythroblastosis [21, 22], little evidence has been obtained for the presence of true neoplastic cells in the Rauscher disease [23, 24]. It has been assumed that the disease would result from the continuous spread of the virus [3]. A breakthrough in the development of the disease will be the accumulation of immature erythroid cells, which do not proliferate at an unlimited rate. This leads to a lack in physiologically mature cells, giving rise to an erythropoietic stimulus, which promotes the development of erythroblastosis.

If this murine leukaemia is a model for some forms of human leukaemia, it is doubtful whether long-standing remissions in these cases can be attributed solely to the eradication of leukaemic blast cells. Fortuitous suppression of the viral genome might be a welcome side effect of the cytotoxic therapy.

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REFERENCES

1. P. J. Fialkow, E. D. Thomas and J. I. Bryant, Leukaemia transformation of engrafted marrow cells *in vivo*. *Lancet* **1,** 251 (1971).
2. S. A. Killmann, Acute leukemia: development, remission/relapse pattern relationship between normal and leukemic hemopoiesis, and the "Sleeper-to-Feeder" stem cell hypothesis. *Ser. Haematol.* **1,** 103 (1968).
3. P. Bentvelzen, A. M. Aarssen and J. Brinkhof, Defectivity of Rauscher murine erythroblastosis virus. *Nature New Biol.* **239,** 123 (1972).
4. J. E. Till and E. A. McCulloch, A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14,** 213 (1961).
5. P. 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).
6. F. J. Rauscher, Virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. *J. nat. Cancer Inst.* **29,** 515 (1962).
7. M. Boiron, J. P. Levey, J. Lasneret and S. Oppenheim, Pathogenesis of Rauscher leukemia. *J. nat. Cancer Inst.* **35,** 865 (1965).
8. I. Brodsky, E. M. Ross, S. B. Kahn and G. Petkov, The effect of a leukemia virus on thrombopoiesis. *Cancer Res.* **28,** 2406 (1968).
9. H.-J. Seidel, Histologische Untersuchungen der Erythropoiese bei 2 Mäusestammen nach Infektion mit dem Rauscher-Virus. *Verh.* 13*e Tagung Deutsche Gesellsch. Haematol. Lehmans Verlag*, p. 119 Munich (1969).
10. D. H. Pluznik, L. Sachs and P. Resnitzky, The mechanism of leukemogenesis by the Rauscher leukemia virus. *Nat. Cancer Inst. Monogr.* **22,** 3 (1966).
11. J. P. O'Kunewick, E. L. Philips and P. Erhard, Increase in number of splenic transplantable colony-forming units in the SJL/J mice after infection with Rauscher leukemia virus. *J. nat. Cancer Inst.* **49,** 1101 (1972).
12. H. -J. Seidel, Hemopoietic stem cells in mice with virus-induced leukemia. II. Studies in C3H mice after Rauscher virus infection. *Z. Krebsf.* **80,** 229 (1973).
13. J. Hajdik, H. Niewisch and G. Matioli, Hematopoietic stem cells from mice with erythroblastosis. *J. Cell. Physiol.* **77,** 393 (1971).
14. F. Wendling, P. E. Tambourin and P. Jullien, Hematopoietic CFU in mice infected by the polycythemia-inducing Friend virus. I. Number of CFU and differentiation pattern in the spleen colonies. *Int. J. Cancer* **9,** 554 (1972).
15. G. Matioli, Friend leukemic mouse stem cell reversion to normal growth in irradiated hosts. *J. reticuloendoth. Soc.* **14,** 380 (1973).

16. A. A. Axelrad and R. A. Steeves, Assay for Friend leukemia virus: Rapid quantitative method based on enumeration of macroscopic spleen foci in mice. *Virology* **24,** 513 (1964).
17. F. Wendling, P. E. Tambourin and P. Jullien, Hematopoietic colony-forming units in mice infected by the polyethemia-inducing Friend virus. III. Pattern of blood recovery in irradiated mice grafted with normal or infected spleen cells. *J. nat. Cancer Inst.* **51,** 179 (1973).
18. G. Bataillon, Sensitivity to guinea-pig serum of hamster cells transformed *in vitro* by two strains of the Rous sarcoma virus. *Int. J. Cancer* **13,** 9 (1974).
19. B. Dunn, R. A. Malmgren, P. C. Carney and A. W. Green, PTK and transfusion modification of the effects of Rauscher virus in BALB/c mice. *J. nat. Cancer Inst.* **36,** 1003 (1966).
20. H. -J. Seidel, Die Blutzellbildung bei der Rauscher Leukämie (Mäusestamm BALB/c) und ihre Beeinflussung durch Hypertransfusion. *Z. schr. Krebsforsch.* **77,** 115 (1972).
21. C. Friend and J. R. Haddad, Tumor formation with transplants of spleen or liver from mice with virus-induced leukemia. *J. nat. Cancer Inst.* **25,** 1279 (1960).
22. S. Thomson and A. A. Axelrad, A quantitative spleen colony assay method for tumor cells induced by Friend leukemia virus infection in mice. *Cancer Res.* **28,** 2105 (1968).
23. P. Bentvelzen, Evidence against the neoplastic nature of virus-induced erythroblastosis in mice. *Europ. J. Cancer* **8,** 584 (1972).
24. E. J. P. Brommer and P. Bentvelzen, Interactions between differentiation and leukaemogenesis in Rauscher murine leukaemia. In *Unifying Concepts of Leukemia.* (Edited by R. M. Dutcher and L. Chieco-Bianchi), p. 929. Karger, Basel (1973).

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Announcements

Institut Jules Bordet
Service of Medicine

A One-day Symposium:

INFECTIONS IN CANCER CHEMOTHERAPY

will be held on January 23, 1975.

Topics to be presented and discussed will include: *pathogenesis* and *diagnosis* of infections in cancer patients; *granulocyte transfusion; protected environment* and *antimicrobials.*

Participants are requested to register at the Institut Jules Bordet, Rue Héger-Bordet, 1, 1000 Bruxelles, Belgium (attention Dr. J. Klastersky, Department of Medicine).

Fourth Annual Conference of the International Society for Experimental Hematology

This Conference will take place in Medena Bay (near Split), Croatia, Yugoslavia, from September 21 to 24, 1975.

The topics will be:

1. Hematopoiesis and the stem cells,
2. Lymphatic tissue—lymphocytes, macrophages, immunity,
3. Bone marrow grafting—experimental and clinical,
4. Experimental neoplasms of the hemopoietic tissue,
5. Experimental models of hematologic disorders,
6. Immune deficiency diseases.

The annual meeting of the Cooperative Group for Bone Marrow Transplantation in Man is planned for September 25.

All further information as well as registration forms may be obtained through correspondence with:

M. Boranić,
Department of Experimental Biology and Medicine,
Rudjer Bošković Institute,
41001 ZAGREB — Yugoslavia,
P.O. Box 1016.

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Erratum

GHANEM ATASSI, H. J. TAGNON and A. TROUET, with the technical assistance of FRANCINE BOURNONVILLE and MICHEL WYNANDS: Comparison of adriamycin with the DNA–adriamycin complex in chemotherapy of L_{1210} leukemia. *Europ. J. Cancer* **10,** 399–403.

The Publishers wish to apologise for the omission of Dr. Trouet's name at the head of the above article.