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Cell-Mediated Immunity in Polyoma Oncogenesis*

M. VANDEPUTTE and S. K. DATTA
Rega Institute for Medical Research, University of Leuven, Belgium

Abstract—*Primary polyoma virus-induced tumors in rats were prevented by the transfer of sensitized lymphoid cells. Sensitized lymphoid cells from donors infected with the virus or immunized with allogeneic polyoma tumors were both effective. The efficacy of the transfer gradually decreased with the increase in the period of time between the virus inoculation and the injection of the immune cells. Non-sensitized lymphoid cells were only effective when given before the virus inoculation. By this* in vivo *transfer assay the gradual development of the cell-mediated immunity directed against polyoma TSTA could be demonstrated, indicating the absence of tolerance or unresponsiveness to the tumor antigens.*

INTRODUCTION

POLYOMA virus-induced tumors in the rat contain tumor-specific transplantation antigen(s) (TSTA) foreign to the normal tissues of the host [1]. These TSTA evoke in the host an immunologic response, chiefly of the homograft-rejection type, which can be detected *in vivo* as well as *in vitro* [2]. Furthermore, the strength of this cellular immunologic response seems of paramount importance in determining whether or not a neoplastic clone will lead to the development of a clinical tumor. To assess the role of cell-mediated immune mechanisms in polyoma oncogenesis two main approaches are used. One consists in verifying the influence of immunodepression (X-irradiation, thymectomy, ALS) on the tumor incidence or tumor-latency period [3–7]. The alternate approach is to test the tumor repression effectiveness of an increased cellular immunologic response obtained by non-specific stimulation such as BCG [8] or poly I : C [9], or by specific stimulation as achieved by transfer of normal or sensitized lymphoid cells [10–12]. In the present study we tried to evaluate by the transfer of sensitized syngeneic lymphoid cells (a) the period after virus inoculation during which tumor development can be prevented in non-immunosuppressed syngeneic rats, and (b) the appearance of cellular immunity during the process of primary polyoma oncogenesis.

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MATERIAL AND METHODS

Animals

Rats of the inbred strains Wistar R and Brown Norway (BN) were used. They were kept on a standard diet of pellets and water *ad libitum*.

Virus

Polyoma virus was grown on mouse embryo fibroblast cultures (MEF) and prepared by the method of Crawford [13]. The virus was titrated with the plaque method of Dulbecco and Freeman on MEF cells [14].

Transplantable tumors and immunization procedures

Adult female R rats were inoculated 3 times subcutaneously (s.c.) at weekly intervals with a BN transplantable polyoma fibrosarcoma. This tumor was free of demonstrable virus as determined by the absence of hemagglutination-inhibiting antibodies in the recipients and by *in vitro* culturing. Control R rats were immunized with an allogeneic (BN) transplantable 7,12-dimethylbenz(a)anthracene (DMBA) sar-

coma following the same schedule as for the BN polyoma tumor.

Five to six days after the last immunization, dissociated lymphoid cells were obtained from the spleen and the combined cervical, inguinal and axillary lymph nodes and prepared by the method described elsewhere [2]. The lymphoid cells were counted and the percentage of living cells determined by trypan blue exclusion. A suspension was made up in Eagle medium to contain 10×10^6–100×10^6 living lymphoid cells in 0·1ml and was inoculated either intraperitoneally (i.p.) or intravenously (i.v.) in the lateral tail vein at the concentration of $2–5 \times 10^6$ cells/g of body weight. Serum was obtained by cardiac puncture from the same immunized syngeneic donor rats. The sera were stored at −20°C. Another group of adult female R rats was immunized by high doses of polyoma virus, having a titer of 1×10^9 plaque-forming units (PFU) per ml, administered s.c. on the right and left flank alternatively and i.p. twice a week for approximately 8 weeks. Lymph nodes and spleen were removed 4 to 6 days after the last inoculation and processed as described above. The serum obtained by cardiac puncture was inactivated for ½ hr at 56°C. This hyperimmune serum had a titer of 1 : 16·384, as determined by the hemagglutination-inhibition test [15].

Histology

Eight to nine weeks after inoculation all surviving rats were killed after bleeding by cardiac puncture. Rats developing clinical signs of tumor growth before this period were processed in the same way. The kidney and brain, as well as the lymphoid organs, were fixed in Bouin's fluid and further processed for histological examination.

RESULTS

Transfer of lymphoid cells or serum from polyoma virus immunized syngeneic donors

Litters of R rats were inoculated i.p. with 5×10^6 PFU of polyoma virus, 5 days after birth. They were divided into different experimental groups depending upon the time interval after birth or virus inoculation when transfer of lymphoid cells or hyperimmune serum was effected.

The results shown in Table 1 indicate that the transfer of 2×10^6 sensitized (immunized) lymphoid cells per gram of body weight protected the recipients against tumor development when the transfer was effected either before (group 1A) or 8 days after virus inoculation (group 2A). Sensitized lymphoid cells transferred 14 or 21 days after virus inoculation had no protective effect. Normal non-sensitized lymphoid cells were effective only when given neonatally and at the level of 5×10^6 cells/g of body weight (group 1C).

Hyperimmune serum injected i.p. in 0·1 ml amounts at 1, 3, and 4 days after birth was very effective in preventing tumor development (group 5). Serum treatment started 5 days after the virus inoculation (i.e. 10 days after birth); however, it was totally ineffective (group 6).

Transfer of lymphoid cells from syngeneic donors immunized by allogeneic polyoma tumor or allogeneic DMBA tumor

This experiment was carried out to check the effectiveness of lymphoid cells sensitized against polyoma TSTA or unrelated (DMBA) TSTA in the prevention of the induction of polyoma tumors in rats.

The design of the experiment was the same as in the previous one. Yet to increase the sensitivity of the system the recipients were infected with a lower dose of virus, 10^6 PFU instead of 5×10^6 PFU and the number of transferred lymphoid cells was increased from 2×10^6–5×10^6/g of body weight in the recipients treated 14 and 21 days after virus inoculation.

As shown in Table 2, a significant reduction in the tumor incidence was observed in all the experimental groups receiving sensitized (anti-polyoma TSTA) lymphoid cells. However, the reduction gradually decreased with the increase of the time period between virus infection and transfer of lymphoid cells. The transfer of lymphoid cells not sensitized against polyoma TSTA was only effective when administered neonatally and had no protective effect given after the virus inoculation.

The serum obtained from the donors of anti-polyoma TSTA sensitized lymphoid cells and inoculated i.p. in 0·2 ml amounts at 5, 7, and 9 days after the virus inoculation had no protective effect. This serum did not contain anti-polyoma virus antibodies (HAI titer < 1/120).

In the groups transferred with DMBA tumor sensitized lymphoid cells and, in the control group, about 10% of the rats (11/128) had to be killed, having clinical symptoms of tumor development before the end of the experiment (8–9 weeks after virus inoculation). In the groups treated with anti-polyoma TSTA sensitized lymphoid cells, 3 out of 105 rats were autopsied with tumors 5 to 6 weeks after the virus inoculation. As the overall tumor incidence was also much lower in the experimental groups compared to the control groups. one cannot conclude from these small numbers that the tumor progression was slower in rats transferred

Table 1. Effect of transfer of lymph node and spleen cells (LN and SC) from immunized (polyoma virus) and non-immunized (normal) syngeneic donors on the development of polyoma tumors in R rat

Groups		Time, dose and route of inoculation of LN and SC	Type of LN and SC inoculated	Number inoculated	Number autopsied	Number and % with tumors	Tumor types			Significance of the tumor reduction
No.	Sub-groups						Cerebr. hemang.	Kidney sarc.	Osteo sarc.	
1	A	0–1 day after birth 2×10^6/g body weight, i.p.	sensitized	26	26	11(42·3%)	9	6	5	$P < 0.001$
	B		non-sensit.	24	22	20(90·9%)	17	19	18	—
	C	5×10^6/g body weight i.p.	non-sensit.	22	22	16(72·7%)	9	15	10	$P < 0.05$
2	A	8 days after virus infection 2×10^6/g body weight i.v.	sensitized	13	13	2(15·9%)	2	0	0	$P < 0.001$
	B		non-sensit.	15	15	15(100%)	12	15	5	—
	C	5×10^6/g body weight i.v.	non-sensit.	16	16	13(92·4%)	11	13	7	Non-signif.
3	A	14 days after virus infection 2×10^6/g body weight i.v.	sensitized	19	19	17(89·4%)	15	14	0	Non-signif.
	B		non-sensit.	18	17	15(88·2%)	14	15	3	—
4	A	21 days after virus infection 2×10^6/g body weight i.v.	sensitized	16	17	16(100%)	16	16	4	—
	B		non-sensit.	15	14	14(100%)	14	14	5	—
5		Immune serum 1, 3 and 4 days after birth, 0·1 ml i.p.	none	21	20	4(20%)	2	4	1	$P < 0.001$
6		Immune serum 10, 12 and 15 days after birth, 0·2 ml i.p.	none	20	20	19(95%)	11	18	9	—
7		Control	none	36	33	31(94%)	29	31	13	—

All rats were inoculated intraperitoneally 5 days after birth with 5×10^6 PFU polyoma virus.

Table 2. Effect of transfer of lymph node and spleen cells (LN and SC) from syngeneic donors immunized by allogeneic polyoma tumors or allogeneic DMBA tumor on the development of polyoma tumors in R rat

Groups		Time, dose and route of inoculation of LN and SC	Type of LN and SC inoculated	Number inoculated	Number autopsied	Number and % with tumors	Tumor types			Significance of the tumor reduction
No.	Sub-group						Cerebr. hemang.	Kidney sarc.	Osteo sarc.	
1	A	0–1 day after birth 5×10^6/g body weight, i.p.	polyoma-sensitized	29	29	4(13·7%)	4	1	0	$P < 0{\cdot}001$
	B		DMBA-sensitized	24	24	14(58·3%)	10	9	17	$P < 0{\cdot}005$
2	A	8 days after virus infection 2×10^6/g body weight, i.v.	polyoma-sensitized	29	29	8(27·5%)	4	8	1	$P < 0{\cdot}001$
	B		DMBA-sensitized	26	26	21(80·7%)	16	15	17	Non-signif.
3	A	14 days after virus infection 5×10^6/g body weight, i.v.	polyoma-sensitized	25	25	16(64%)	8	15	3	$P < 0{\cdot}01$
	B		DMBA-sensitized	21	21	16(76·2%)	12	15	10	Non-signif.
4	A	21 days after virus infection 5×10^6/g body weight, i.v.	polyoma-sensitized	22	22	16(72·7%)	10	15	14	$P < 0{\cdot}05$
	B		DMBA-sensitized	26	26	24(92·3%)	17	20	20	Non-signif.
5		Serum 10, 12 and 15 days after birth, 0·2 ml i.p.	none	25	25	24(96%)	20	23	16	—
6		Control	none	32	31	29(93·5%)	20	29	23	—

All rats were inoculated intraperitoneally 5 days after birth with 10^6 PFU of polyoma virus.

with anti-polyoma TSTA sensitized lymphoid cells.

The histopathological examination of the tumors did not reveal differences in tumor histology between the experimental and the control groups. In only two cases (1 rat in group 1A and 1 rat in group 2B) did the histology reveal neoplastic foci in the kidney which had not been noted at the macroscopic examination as tumor.

Development of anti-polyoma TSTA cellular immunity during the process of polyoma oncogenesis in rats

To verify the development of cellular immunity directed against polyoma TSTA during the process of primary polyoma oncogenesis, we used the same experimental model as described above. The rats (donor animals) in which the appearance of this cellular mediated immunity was verified were inoculated 3 days after birth i.p. with 5×10^6 PFU polyoma virus. Previous experiments had shown that such a dose induced polyoma tumors in 95–100% of the animals.

Lymphoid cells were prepared from these donors respectively 6, 15, and 21 days after the virus inoculation and transferred i.v. into 13-day old syngeneic recipients rats inoculated 8 days previously at the age of 5 days with 10^6 PFU polyoma virus i.p.

We chose the experimental model of transferring the lymphoid cells into recipients inoculated 8 days before with the virus for the following reasons: (a) as shown in the previous experiments, the transfer of lymphoid cells 8 days after virus inoculation is a very sensitive system to detect antipolyoma TSTA cellular immunity; (b) the protective effect of the transferred lymphoid cells, if present, cannot be caused by an antiviral action; (c) 13-day old recipients do not necessitate as many donors as older recipient animals would do. Taking into account the number of lymphoid cells required for transfer and the relatively small numbers recovered from young donors transfer into older animals would require too great a number of donors; (d) normal, non-sensitized lymphoid cells do not exert any protective effect when transferred after polyoma virus inoculation.

The results of this experiment, as shown in Table 3, indicate that recipient rats were significantly protected by lymphoid cells removed from donors 15 or 21 days after polyoma virus inoculation. Donor lymphoid cells removed earlier, e.g. 6 days after virus inoculation, were apparently not able to protect the recipients.

DISCUSSION

Our results illustrate the efficacy of syngeneic lymphoid cells sensitized against polyoma TSTA (by allogeneic polyoma tumor cells) in the prevention of primary polyoma virus induced neoplasms. This is in accordance with the results obtained in immunosuppressed mice injected with polyoma virus or with Moloney sarcoma virus and afterwards immunologically restored with sensitized lymphoid cells [12, 16, 17]. Transfer of sensitized thoracic duct cells was also effective in protecting against chemical carcinogen induced tumors [18]. However, in the latter case the experiments were carried out in a non-syngeneic system.

Whereas lymphoid cells from donors sensitized with allogeneic polyoma tumors gave a good protection, the serum from the same animals was ineffective.

Lymphoid cells from donors immunized with polyoma virus were also found to be effective, whether transferred before or 8 days after the virus inoculation. In the latter case their efficacy cannot be due to a transfer of an antiviral serum-mediated immunity as shown by the inability of high doses of hyperimmune anti-polyoma virus antiserum to inhibit the tumor development when injected after the virus. Moreover, previous studies have shown that in the rat the virus persists only for a very short period of time after its inoculation [6]. Hence, it is very likely that the polyoma virus inoculated into the donor rats induces both antiviral humoral response and an immunologic reaction directed against the virus induced TSTA. Evidence for this is also given by the resistance against syngeneic polyoma tumor grafts found in animals pretreated with the virus [1].

Although lymphoid cells sensitized against polyoma TSTA seem to give a better protection than lymphoid cells from donors inoculated with the virus, no definite conclusion can be drawn from our experiments as the dose of virus and the number of lymphoid cells transferred were different in the two experiments.

The explanation of this protective effect could be that administration of sensitized, actively immune lymphoid cells in polyoma virus infected rats, upon seeding out and becoming established in the new host, increased their immunologic reactivity of cell mediated type, and thus enabled them to mount an effective immune response against arising neoplastic clones. But, there seems to be a critical limit of the size of neoplastic cell mass, which if attained during oncogenesis, outflanks the efficient rejection responses of the host, even if they are

Table 3. Effect of transfer of lymph node and spleen cells (LN and SC) from polyoma virus-inoculated rats on the development of tumors in syngeneic recipients

Groups	Time, dose and route of inoculation of LN and SC	Donor type of LN and SC inoculated	Number inoculated	Number autopsied	Number and % with tumors	Tumor types			Significance of the tumor reduction
						Cerebr. hemang.	Kidney sarc.	Osteo sarc.	
1	8 days after virus infection, 2×10^6/g body weight, i.v.	6 days after P.V. inoculation	20	19	11(58%)	6	10	9	Non-signif.
2	8 days after virus infection, 2×10^6/g body weight, i.v.	15 days after P.V. inoculation	12	12	2(17%)	2	2	2	$P < 0{\cdot}01$
3	8 days after virus infection, 2×10^6/g body weight, i.v.	21 days after P.V. inoculation	12	12	4(33·3%)	1	5	3	$P < 0{\cdot}02$
4	Controls	none	42	42	31(73·8%)	20	23	26	—

Donor rats were inoculated i.p. 3 days after birth with 5×10^6 PFU polyoma virus (P.V.).
Recipient rats were inoculated i.p. 5 days after birth with 10^6 PFU polyoma virus.

strengthened through passive transfer of sensitized lymphoid cells, and leads to progressive development of tumor. This is indicated by the decrease in efficacy as the period of transfer of sensitized lymphoid cells, after virus infection is gradually increased (Table 2).

Lymphoid cells sensitized against an antigenic unrelated DMBA tumor as well as control lymphoid cells were only active when inoculated before the virus and at a very high dose level (Tables 1 and 2). Presumably, the control lymphoid cells transferred soon after birth, after getting adjusted to new environment, need enough time to get sensitized and take part in cell-mediated reaction to the stimulus provided by the TSTA of the neoplastic clones. Because in our experiments the transfer was done in normal rats, the results are difficult to compare with those obtained in thymectomized animals in which non-sensitized normal lymphoid cells were also found to be effective after the virus inoculation [16]. Moreover, in the latter case, a strain of mice was used which is only sensitive to polyoma oncogenesis when under immunosuppressive treatment.

It may be concluded that in the process of oncogenesis, the animals which display efficient cell mediated responses resist the development of tumor, whereas others which fail to acquire enough immunological reactivity succumb to tumorous state. Whether serum factor(s), which block the inhibitory effect of sensitized lymphocytes *in vitro* and thereby nullify the useful effect of cell-mediated immune reaction, also play an important role *in vivo* is still an open question [2, 19]. The clinical outcome seems to be based on a delicate balance in which the strength and the rapidity in the development of an anti-TSTA cell-mediated immunity are of paramount importance. From the results obtained with transferred lymphoid cells, prepared from donors at different times after the various inoculation, it is clear that the cell-mediated anti-TSTA immunity is gradually built up and is easily demonstrable 2 and 3 weeks after the virus inoculation (Table 3). Moreover, there exists a good correlation between the demonstration of antipolyoma TSTA sensitized cells as assayed by the *in vivo* transfer system and by the colony-inhibition test *in vitro* [unpublished observation]. As shown by Allison [20], the much more rapid development of an efficient cell-mediated immunity in adults compared to newborn animals may well be the main reason why the former are resistant to polyoma oncogenesis. However, it is clear that in neither case is there any evidence of tolerance or unresponsiveness to the polyoma TSTA.

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Properties of Transplantation Antigen present in Cell-Free Extracts of Adenovirus-12 Tumours

E. G. BROWN, C. W. POTTER and J. S. OXFORD

Department of Medical Microbiology, University of Sheffield, and Virus Research Laboratory, Lodge Moor Hospital, Sheffield, England

Abstract—*Mice which had been immunized with cell-free extracts of transplanted adenovirus 12-induced tumours of CBA mice were relatively immune to subsequent challenge with viable, homologous tumour cells. The immunogenicity of these tumour cell extracts was unaffected by heating at* 37°C *for* 1 *hr or* 56° C *for* 30 *min. Immunization of mice with tumour cell extracts which had been treated with* 0·5% *trypsin did not induce transplantation immunity, but the tumour cell extracts were active after treatment with DNase or RNase. Treatment with* 0·5% *DOC,* 0·5% *SDS or* 1·0% *Tween* 80 *destroyed the immunogenicity of the tumour cell extract. Transplantation immunity was present in mice immunized with extracts which had been treated with ether, but immunity was not demonstrable in mice immunized with extracts which had been treated with lipase or phospholipase. The transplantation antigen was precipitated by dialysis at pH* 5·0–6·0, *and was not recoverable from the precipitate following re-dialysis at pH* 7·4.

INTRODUCTION

THE CELLS of tumours induced by adenovirus 12 have been shown to contain a virus-specific transplantation immunity antigen [1], since these cells were rejected by animals previously immunized with either adenovirus 12 or killed, intact homologous tumour cells [2, 3]. Similar antigens have been described in the cells of tumours induced by other viruses including orphan virus [6]. Earlier reports indicated that transplantation immunity induced by the inoculation of tumour cells required that the tumour cells were intact [3, 7, 8]. However, recent studies have reported transplantation immunity in animals immunized with tumour cell fragments [9–11], or with "soluble" extracts of tumour cells [12]. Whether the antigens are truly soluble in these preparations or attached to cell membrane fragments remains to be determined.

In the present study, we report some of the properties of transplantation immunity antigen present in extracts from transplanted adenovirus 12-induced tumours of CBA mice. Cell extracts were treated with a variety of enzymes and chemicals and under various physical conditions, and then used to immunize groups of CBA mice. The mice were subsequently challenged with viable tumour cells. Rejection of the tumour cells indicated that transplantation immunity was conferred by immunization, while the absence of demonstrable immunity indicated that the treatment had destroyed the antigen, or destroyed a carrier structure or molecule necessary for antibody induction. These tests would indicate some properties of the transplantation immunity antigen which would help in the design of techniques for purification procedures, and which would aid further studies of the nature and specificities of the antigen.

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METHODS AND MATERIALS

Adenovirus-12 tumour cells

Groups of CBA mice bearing transplanted adenovirus 12-induced tumours were killed when tumours had grown to a diameter of 10–15 mm. The tumours were removed, freed of adhering tissues and minced to small fragments. Fragmented tumours from 4–6 mice were pooled and treated with 0·25% trypsin to produce a single cell suspension. The cells were washed with two changes of Eagle's minimal essential medium containing 2% inactivated calf serum, suspended in the same medium at a concentration of $5{\cdot}0 \times 10^6$ viable cells/ml and used immediately. All mice were inoculated subcutaneously with 0·1 ml of tumour cells; this number of cells had been observed to produce tumours regularly within 5 weeks of inoculation in 90–100% of mice, and represented ten (50%) tumour cell doses.

Preparation of antigen extracts

Adenovirus 12-induced tumours from groups of 10–30 CBA mice were harvested and fragmented as before. The tumour fragments were washed with Hanks' saline and centrifuged at 2000 rev/min for 15 min. The pellets of tumour fragments were resuspended in Hanks' saline to a concentration of 20% (v/v), briefly homogenized in a Waring blender and frozen and thawed (−80°C/22°C). The disrupted tumour cell preparation was centrifuged at 100,000 **g** for 1 hr, and the supernatant fluid filtered through a Millipore filter of 0·5μ pore size. These preparations, which contained 1·2–1·5 mg/ml protein [13] represented the extracts of approximately $3{\cdot}0 \times 10^6$ tumour cells/0·1 ml and were stored at −80°C in 2 to 5 ml ampoules.

Antigen extracts from normal mouse skeletal muscle tissue were prepared in a manner identical to that described for the preparation of tumour antigen extracts.

Experimental design

Experiments were carried out in CBA mice from a closed colony of randomly mated mice. The immunization schedules were started at age 4 to 5 weeks.

Groups of approximately 20 CBA mice were inoculated subcutaneously and at weekly intervals with three, 0·1 ml doses of an antigen preparation. Mice were immunized with treated tumour cells extracts, and further groups of mice received untreated tumour cell extracts or tumour cell extracts which had been subjected to the physical conditions of treatment only, or normal cell extracts treated in the same manner. Fourteen days after mice had received the third immunizing dose, all mice were challenged by the subcutaneous inoculation of 5×10^5 viable tumour cells in a 0·1 ml volume. Mice were examined weekly for tumours, and mice with tumours of 15 mm or more in diameter were killed. Mice were examined for a period of 5 weeks, since tumours rarely developed after this period. Five weeks after tumour cell challenge the protective effects of immunization with the various treated tumour cell preparations was assessed by comparing the tumour incidence in these mice with that found in CBA mice immunized with control antigen extracts.

Treatment of antigen extracts

1. *Heat stability.* Aliquots of tumour cell extracts and normal mouse tissue extracts were thawed, immersed in a water bath at 37°C for 1 hr, or at 56°C for 30 min, and subsequently used to immunize groups of CBA mice.
2. *Detergent treatment.* Cell extracts were dialysed overnight at 4°C against 100 volumes of 0·5% sodium dodecyl sulphate (BDH Chemicals Ltd., Poole, Dorset), 0·5% sodium deoxycholate (BDH Chemicals Ltd.) or 1·0% Tween 80 (Sigma Chemical Co. Ltd.) dissolved in phosphate buffered saline, pH 7·4 (PBS). The extracts were then re-dialysed against 100 volumes of PBS to remove detergent and then stored in ampoules at −80°C.
3. *Ether treatment.* Cell extracts of tumour cells and of normal mouse tissue were each treated with an equal volume of peroxide-free ether and mixed for 10 min at 4°C. The ether was then removed by aeration with 5% CO_2 in air. Ether treatment was carried out three times, and the preparations stored at −80°C prior to the immunization of groups of CBA mice.
4. *Treatment with enzymes.* Samples of tumour cell extracts and normal mouse tissue extracts were treated with 0·5% (w/v) crystalline trypsin, 200 μg/ml RNase or 200 μg/ml DNase (all from BDH Chemicals Ltd.). The enzymes were dissolved directly into the tumour cell extracts, and incubated at 37°C for 1 hr. Further samples of tumour and normal mouse tissue extracts were treated with 20 μg/ml lipase (wheat germ extract, Calbiochem Ltd., London) or phospholipase (Calbiochem Ltd.) and incubated at 37° for 1 hr at pH 7·4. After enzyme treatment, antigen extracts were stored at −80°C prior to immunization of CBA mice. No attempt was made to neutralize the enzyme activity after the incubation period.
5. *Effect of pH on tumour antigen.* Tumour cell extracts and extracts of normal mouse tissues

were dialysed overnight at 4°C against 0·15 M NaCl buffered to pH 3·0., 4·0 or 5·0 with 0·01 M citrate buffer, or 0·15 M NaCl buffered to pH 6·0 or 7·0 with 0·01 M phosphate buffer. The antigen preparations were then centrifuged at 2000 rev/min for 30 min and the supernatant fluid collected and re-dialysed against PBS and stored at −80°C. The precipitates formed during dialysis at different pH values were suspended in a volume of PBS equal to the original volume of antigen extract and redialysed overnight at 4°C against PBS. The residual precipitates were removed by centrifugation at 2000 rev/min for 30 min and the supernatant fluid stored at −80°C. Each of these preparations were used to immunize groups of CBA mice, prior to challenge with tumour cells.

Table 1. *Effect of heat treatment on adenovirus* 12*-induced transplantation antigen*

Immunizing antigen (treatment)	Incidence of tumours* (weeks after challenge)					Total %
	1	2	3	4	5	
Tumour extract (56°C/30 min)	0/20†	3/20	5/20	7/20	7/20	35
Normal cell extract (56°C/30 min)	0/20	9/20	17/20	19/20	19/20	95
Tumour extract (37°C/60 min)	0/20	2/20	4/20	4/20	5/20	25
Normal cell extract (37°C/60 min)	0/20	7/20	16/20	19/20	20/20	100
Tumour extract (unheated)	0/18	1/18	3/18	4/18	5/18	28
Normal cell extract (unheated)	0/20	15/20	18/20	18/20	19/20	95
Nil	0/19	4/19	17/19	19/19	—	100

*Incidence of tumours in CBA mice immunized with treated cell extracts and challenged with $5{\cdot}0 \times 10^5$ viable tumour cells.

†$\frac{\text{No. of animals with tumours}}{\text{No. of animals inoculated}}$.

RESULTS

Heat stability of adenovirus 12 *transplantation antigen*

The incidence of tumours in mice immunized with extracts from transplanted adenovirus 12 tumours, or from normal tissue of CBA mice, which had been heated at 37°C for 60 min or at 56°C for 30 min and subsequently challenged with 5×10^6 viable tumour cells is shown in Table 1. Mice immunized with heated tumour extracts developed significantly fewer tumours than mice immunized with control, normal mouse tissue extracts [X^2 (Yates' Correction) $\geqslant 13{\cdot}347$; $P < 0{\cdot}001$]. Thus, tumour cell extracts which had been heated at 37°C for 60 min or at 56°C for 30 min both induced transplantation immunity, and the transplantation antigen present in the tumour extracts, therefore, was not inactivated by these heat treatments.

Effect of trypsin, Nase and DNase on adenovirus-12 *transplantation antigen*

The results of experiments to assess the effects of trypsin, RNase and DNase on the immunogenicity of extracts from transplanted adenovirus 12 tumours are shown in Table 2. The ability of tumour cell extracts to induce transplantation immunity was destroyed by treatment with 0·5% trypsin. This result was not due to the inactivation of the antigen by the conditions of trypsin treatment, since antigen extracts incubated at 37°C for 60 min in the absence of trypsin induced transplantation immunity. This finding confirmed a result shown in Table 1. In contrast, RNase and DNase had no effect on the ability of tumour cell extracts to induce transplant immunity, since mice immunized with RNase or DNase-treated tumour extracts showed significant immunity to tumour cell challenge. [X^2 (Yates' correction) $\geqslant 14{\cdot}656$; $P < 0{\cdot}001$].

Table 2. Effect of trypsin and nucleases on adenovirus 12-induced transplantation antigen

Immunizing antigen (treatment)	Incidence of tumours* (weeks after challenge)					Total %
	1	2	3	4	5	
Tumour extract (0·5% trypsin)	0/20†	7/20	16/20	19/20	20/20	100
Normal cell extract (0·5% trypsin)	0/20	6/20	16/20	18/20	19/20	95
Tumour extract (DNase, 200 μg/ml)	0/20	1/20	3/20	4/20	5/20	25
Normal cell extract (DNase, 200 μg/ml)	0/21	8/21	18/20	21/21	—	100
Tumour extract (RNase, 200 μg/ml)	0/20	2/20	3/20	5/20	6/20	30
Normal cell extract (RNase, 200 μg/ml)	0/19	8/19	16/19	18/19	18/19	95
Tumour extract (37°C/60 min)	0/20	2/20	3/20	3/20	4/20	20
Normal cell extract (37°C/60 min)	0/20	9/20	17/20	18/20	19/20	95
Nil	0/21	8/21	17/21	19/21	21/21	100

*Incidence of tumours in CBA mice immunized with treated cell extracts, and challenged with $5{\cdot}0 \times 10^5$ viable tumour cells.

†$\frac{\text{No. of animals with tumours}}{\text{No. of animals inoculated}}$.

Stability of adenovirus 12 transplantation antigens to treatment with detergents

The incidence of tumours in mice immunized with tumour antigen extracts which had been dialysed against 0·5% sodium deoxycholate (DOC), 0·5% sodium dodecyl sulphate (SDS) or 1·0% Tween 80, and subsequently challenged with tumour cells is shown in Table 3. In all three cases, the incidence of tumours in mice immunized with treated tumour cell extracts was similar to that observed in mice immunized with control cell preparations. In addition, the immunogenicity of the original tumour cell extract was not inactivated by the conditions of treatment with detergents, as mice immunized with tumour cell preparations which have been dialysed twice against PBS only were relatively immune to tumour cell challenge. This result indicated that 0·5% SDS, 0·5% DOC and 1·0% Tween 80 each destroyed the immunogenicity of the tumour cell preparations.

Stability of antigen to treatment with ether, lipase and phospholipase

Groups of CBA mice were immunized with extracts from transplanted adenovirus 12 tumours which had been previously treated with lipase (20 mg/ml), phospholipase (20 mg/ml) or by extraction with ether (X3). Mice were also immunized with extracts of normal mouse tissue treated in a similar manner. The incidence of tumours in the mice following challenge with viable tumour cells is given in Table 4. Transplantation immunity was not demonstrable for CBA mice immunized with tumour antigen which had been treated with lipase or phospholipase.

Immunization with tumour antigen extracts which had been three times extracted with ether induced transplantation immunity [X^2 (Yates' Correction) = 10·717; $P < 0{\cdot}001$]. Thus, ether extraction did not destroy the immunogenicity of tumour cell extracts,

Table 3. Effect of detergents on adenovirus 12-induced transplantation antigen

Immunizing antigen (treatment)	Incidence of tumours* (weeks after challenge)					Total %
	1	2	3	4	5	
Tumour extract (0·5% SDS)	0/19†	6/19	13/19	11/19	17/19	89
Normal cell extract (0·5% SDS)	0/20	7/20	17/20	19/20	20/20	100
Tumour extract (0·5% DOC)	0/19	6/19	15/19	18/19	18/19	95
Normal cell extract (0·5% DOC)	0/21	5/21	17/21	18/21	19/21	90
Tumour extract (1·0% Tween 80)	0/20	12/20	18/20	19/20	19/20	95
Normal cell extract (1·0% Tween 80)	1/20	13/20	17/20	19/20	20/20	100
Tumour extract (PBS)	0/19	2/19	3/19	5/19	5/19	26
Normal cell extract (PBS)	1/19	5/19	16/19	17/19	17/19	89
Nil	0/20	2/20	16/20	20/20	—	100

*Incidence of tumours in CBA mice immunized with treated cell extracts, and challenged with $5{\cdot}0 \times 10^5$ viable tumour cells.

†$\frac{\text{No. of animals with tumours}}{\text{No. of animals inoculated}}$.

Table 4. Effect of ether and lipases on adenovirus 12-induced transplantation antigen

Immunizing antigen (treatment)	Incidence of tumours* (weeks after challenge)					Total %
	1	2	3	4	5	
Tumour extract (ether *X*3)	0/20†	3/20	7/20	7/20	8/20	40
Normal cell extract (ether *X*3)	0/19	10/19	16/19	18/19	18/19	95
Tumour extract (20 mg/ml lipase)	0/19	9/19	16/19	17/19	17/19	90
Normal cell extract (20 mg/ml lipase)	0/20	11/20	17/20	19/20	19/20	95
Tumour extract (20 mg/ml phospholipase)	0/20	11/20	17/20	18/20	19/20	95
Normal cell extract (20 mg/ml phospholipase)	0/19	12/19	17/19	18/19	18/20	95
Tumour extract (37°C/1 hr)	0/20	2/20	4/20	6/20	7/20	35
Tumour extract (untreated)	0/18	1/18	4/18	5/18	6/18	33
Normal cell extract (untreated)	0/20	13/20	18/20	20/20	—	100
Nil	0/20	17/20	19/20	19/20	20/20	100

*Incidence of tumours in CBA mice immunized with treated cell extracts, and challenged with $5{\cdot}0 \times 10^5$ tumour cells.

†$\frac{\text{No. of animals with tumours}}{\text{No. of animals inoculated}}$.

Table 5. Effect of pH on adenovirus 12-induced transplantation antigen

Tumour antigen dialysed at:—	Incidence of tumours* (weeks after challenge) 1	2	3	4	5	Test Total %	Control† Total %
pH 7·0 supernatant	0/19‡	2/19	2/19	4/19	5/19	20	19/20 (95)
precipitate	0/18	11/18	14/18	15/18	16/18	89	19/19 (100)
pH 6·0 supernatant	0/18	1/18	3/18	4/18	5/18	28	20/20 (100)
precipitate	0/20	12/20	16/20	18/20	18/20	90	18/19 (94)
pH 5·0 supernatant	0/20	11/20	17/20	19/20	19/20	95	18/19 (94)
precipitate	1/19	12/19	16/19	18/19	19/19	100	19/19 (100)
pH 4·0 supernatant	1/19	12/19	17/19	19/19	—	100	18/19 (94)
precipitate	0/18	10/18	15/18	16/18	17/18	94	20/20 (100)
pH 3·0 supernatant	0/19	13/19	18/19	18/19	18/19	95	19/20 (95)
precipitate	1/20	13/20	16/20	18/20	20/20	100	19/19 (100)
Tumour antigen (untreated)	0/20	1/20	3/20	4/20	5/20	25	18/19 (94)
nil	1/19	12/19	18/19	18/19	19/19	100	

*Incidence of tumours in CBA mice immunized with cell extracts exposed to various pH values and challenged with $5{\cdot}0 \times 10^5$ viable tumour cells.

†Total incidence of tumours (%) in CBA mice immunized with extracts from normal mouse tissue, treated in parallel with tumour extracts.

‡ $\frac{\text{No. of animals with tumours}}{\text{No. of animals inoculated}}$.

whereas treatment with lipase and phospholipase and treatment with SDS and DOC (Table 3), which under the conditions used was mainly directed against lipids in the tumour extracts, all resulted in inactivation of transplantation antigen.

Stability of transplantation immunity antigen at different pH values

Groups of CBA mice were immunized with the supernatant fluid or the reconstituted precipitate formed following dialysis of tumour cell extracts at different pH values. Further groups of mice were immunized with comparable preparations from cell extracts of normal mouse tissue. The incidence of tumours in these mice, following challenge with $5{\cdot}0 \times 10^5$ tumour cells, is shown in Table 5. Transplantation immunity was found in mice previously immunized with supernatants from tumour cell extracts dialysed at pH 6·0 and pH 7·0. However, immunization with supernatant fluids collected following dialysis at pH 5·0 or lower did not induce transplantation immunity (Table 5).

The precipitates produced by dialysis at different pH values were collected, diluted to the original volume and dialysed against PBS. No visible precipitate was produced following dialysis of tumour extracts at pH 7·0, but the procedure was followed as if a precipitate had been produced. Mice were immunized with reconstituted precipitates after the irreversible precipitate was removed, and the mice subsequently challenged with tumour cells. In no instance did immunization with reconstituted precipitates induce transplantation immunity (Table 5). Thus, the antigen(s) responsible for transplantation immunity were precipitated or inactivated at pH 5–6 or lower, and could not be subsequently recovered from the precipitate.

DISCUSSION

The present results extended our previous observations that transplantation immunity can be demonstrated following immunization of CBA mice with extracts of adenovirus 12 tumour cells [11]. The extracts were from tumours which had been minced, frozen and thawed, centrifuged at 100,000 **g** for 1 hr and the supernatant passed through a filter of pore size 0·45 μ. Earlier studies had reported that transplantation immunity to DNA virus-

induced tumour cells was limited to animals immunized with homologous virus or whole tumour cells [3, 5]; however, more recent studies have demonstrated transplantation immunity in animals immunized with tumour cell fractions. Thus, ghosts of SV40 virus-induced tumour cells, produced by treating cells with hypotonic solutions [9], and crude membrane fragments from SV40 or adenovirus 31-tumour cells [8], have been reported to induce transplantation immunity. Similarly, immunization with frozen and thawed extracts of adenovirus 7-induced tumour cells [14] or soluble extracts produced by papain treatment of SV40 tumour cell membranes [12] have been shown to induce transplantation immunity against homologous tumour cell challenge. The procedure used in the present study was similar to that used to produce "soluble" transplantation antigens from chemically induced tumours of guinea pigs [15]. However, these procedures may not give soluble antigen preparations, for cell membrane fragments have been reported to occur in supernatant fluids following the centrifugation of cell extracts at 100,000 **g** [16].

In order that further studies can be carried out on the chemical nature, properties and specificities of the adenovirus 12-induced transplantation antigen(s), it is necessary to have the antigen in true solution. In addition, any techniques used to produce such solutions should not alter the natural structure of the antigen. We have examined the stability of the antigen to some physical and chemical agents in order to determine the chemical nature of the transplantation antigen; to indicate possible methods for preparing solutions; and to indicate properties useful for purification studies.

The immunogenicity of adenovirus 12-induced tumour cell extracts was destroyed by treatment with 0·5% trypsin, but resistant to the action of DNase and RNase. These results suggest that the antigen has a protein determinant, and is not dependent on nucleic acid for antigenic specificity. Treatment of the antigen with detergents such as SDS and DOC destroyed the property of tumour cell extracts to induce transplantation immunity, as did treatment with lipase and phospholipase. The antigen was resistant to extraction with ether. These findings can be interpreted in two ways. Firstly, the results suggest that the antigen responsible for the induction of transplantation immunity was a lipoprotein unaffected by ether treatment, and, together with the established effect of trypsin, that the antigenic determinants were residual in both lipid and protein components. Some studies of naturally occurring transplantation antigens have reported that they are lipoprotein in nature, but other studies have shown that further purification gives material which is both antigenic and immunogenic and contains little or no lipid [7]. The second interpretation is that the action of detergents, lipase and phospholipase on tumour cell extracts may be to destroy a lipid carrier or a lipid grouping responsible for antigenic determination and thus to affect the immunogenicity of the transplantation antigen. Further tests are required to test the antigenicity of lipase or phospholipase-treated cell extracts, since the ability of these extracts to induce tumour cell immunity may be reduced rather than lost, and may be demonstrable using a different immunization schedule. Preliminary studies using Freund's complete adjuvant to enhance the immune-response to tumour cell extracts were not successful, as immunization with Freund's adjuvant by itself was found to enhance tumour formation.

The adenovirus 12-induced transplantation antigen of CBA mice was precipitated at pH 5·0–6·0 and the antigen was not recoverable from the precipitate by dialysis at pH 7·4. Thus, the precipitation of the antigen appeared to be either irreversible or the antigen was denatured at pH 5·0–6·0. It is clear that acid precipitation is not a procedure likely to be of value in the purification of the adenovirus 12-induced transplantation antigen.

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Defibrination and Metastasis Formation:

Effects of Arvin on Experimental Metastases in Mice*

B. HAGMAR

Department of Pathology, University of Göteborg, Göteborg, Sweden

Abstract—*The effect of arvin (purified enzyme fraction of Malayan pit viper venom) was tested on two strains of inbred mice. Although the mice were relatively resistant to arvin, a coagulation defect could be induced, which was largely due to fibrinogen depletion.*

The influence of single and multiple dose arvin treatment was tested on intravenously induced metastases from syngeneic sarcoma MCG1-SS in CBA and melanoma B16 in C57 mice. Single dose arvin treatment increased the crop of MCG1-SS metastases in the liver and B16 metastases in other extrapulmonary organs. Multiple dose treatment caused only minor alterations in metastasis patterns.

The results offer evidence against the alleged importance of fibrin as the major factor behind tumour cell retention in organs and metastasis development.

INTRODUCTION

IT HAS been stated that fibrin around disseminated tumour cells constitutes a promoting or essential factor in the development of metastases [1–4]. This notion has not been corroborated in recent experiments with anticoagulants in syngeneic tumour-host systems [3, 4]. But even in such systems anticoagulants alter the distribution of metastases. Diverging results have been obtained, however, with different anticoagulants, e.g. heparin and coumarin. This finding has raised the question whether anticoagulants may affect metastasis formation by mechanisms other than impaired blood coagulability [4, 5].

Defibrination of experimental animals subjected to intravenous injection of tumour cells would help to define the role, if any, of fibrin in metastasis development. Defibrination has been achieved in man and in some animal species with a purified fraction of Malayan pit viper venom, "arvin" [6]. It has a thrombin-like action, precipitating a non-stabilized type of fibrin intravascularly which is removed by fibrinolysis and RES activity [7–10].

It was the aim of the present investigation to test the effect of arvin on experimental metastases. Two murine syngeneic tumour-host systems were used and the effects of arvin in single and multiple dose treatment were compared. The single dose treatment should demonstrate what defibrination means for the initial retention of tumour cells in vessels. The multiple dose treatment should also cover the initial growth of metastases.

Since there are considerable species-differences in arvin susceptibility [11] and the coagulation defect induced in mice seems not to have been described previously, the study had to start by determining appropriate dosages for the mice strains used.

MATERIAL AND METHODS

The studies were performed in two strains of inbred mice: CBA and C57 BL/6J. The animals

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were 2–3 months old when taken for experiments and weighed approximately 20 g.

Arvin (kindly supplied by Twyford Laboratories Ltd., London) is a chromatographically purified enzyme fraction of the Malayan pit viper (*Agkistrodon rhodostoma*) venom [6]. It is standardized to correspond unit for unit to thrombin [10]. Fresh solutions in saline containing 50 units/ml were prepared each experimental day. The doses were adjusted to body weights, given intraperitoneally or intravenously by a tail vein. Controls were given a corresponding volume of saline. To obtain exact dosages 100 µl microsyringes were used.

Coagulation tests

In several series of mice, with or without previous tumour cell injection, the following parameters were tested.

Whole blood clotting time. The capillary method [12] was used on blood obtained by cutting the tip of the tail. Mean values (±S.D.) were in 30 CBA mice 180 (±50) sec and in 30 C57 mice 155 (±44) sec.

In some initial experiments the method of Lee and White [13] was used. It was abandoned, not only because of the larger amounts of blood needed, but also because the small and fragile clots from arvin-treated animals were often difficult to detect.

Thrombin clotting time of plasma. Blood (0·9 ml) was drawn from the right side of the heart into a syringe containing 0·2 ml of a mixture 1 : 1 of 3·8% trisodium citrate and 10% ε-aminocaproic acid (EACA) in saline. After centrifugation the plasma was withdrawn and the thrombin time determined without delay to avoid non-specific prolongation of the clotting time [4]. Thrombin (Topostasin, F. Hoffman-La Roche and Co AG, Basel, Switzerland) was diluted on the experimental day with saline-tris buffer (4 : 1; pH 7·5) to contain 5 NIH-units/ml.

The test was performed similarly to the method described by Triantaphyllopoulos and Triantaphyllopoulos [14] with and without the presence of a standard solution human fibrinogen in order to obtain presumptive evidence for the presence of fibrinogen degradation products (FDP) with anticoagulant properties. The test had to be performed with small volumes of plasma, since the same samples were used for fibrinogen determinations (*vide infra*). Thus, with disposable 20 µl micropipettes 40µl plasma was transferred to a small plastic test tube, 20 µl thrombin solution was quickly added, the contents mixed and the clotting time at room temperature determined. A steel wire loop was used to detect the formation of a coherent fibrin clot.

A parallel test was performed with the same plasma, 20 µl being mixed before addition of thrombin with 20 µl standard fibrinogen solution (100 mg% human fibrinogen, Grade L, 88% clottable, kindly supplied by AB Kabi, Stockholm, Sweden). When performed with standard fibrinogen alone the coagulation time was uniformly 18 sec.

Fibrinogen. Plasma was obtained as described in the preceding paragraph. The thrombin-clottable fibrinogen was determined by a modification of the Ratnoff and Menzie procedure [15]. For the initial steps the solutions and procedures described by Huseby and Bang [16] were used, but with a coagulation time of 1 hr. To increase the sensitivity in the low test interval of interest here, the glycine-buffer after dissolution of the clot in boiling NaOH was abandoned. Instead the NaOH after cooling was diluted with 3·5 ml re-distilled water, 1·5 ml 20% sodium carbonate and 0·5 ml Folin–Ciocalteu reagent. These are the solutions of the original method, but here used in half volumes.

Plasma samples of 0·4 ml were used, except when a PS-test (*vide infra*) was simultaneously performed, when 0·2 ml was used for each type of determination. The method was standardized against human fibrinogen (AB Kabi, *vide supra*). A linear calibration curve through the origin was obtained with the above described modifications in the range 0–300 mg%. Each test day three points on the calibration curve were checked (25, 50 and 100 mg%). Great care had to be taken that the fine strands of fibrin were caught at the very moment of coagulation, since otherwise falsely low values were obtained.

The *protamine sulfate (PS) test* was performed according to Bell *et al.* [17] with 10 mg% PS (kindly supplied by Vitrum AB, Stockholm, Sweden) in the saline-calcium cloride-EACA solution used to dilute plasma before addition of thrombin. The samples were stored for 1 hr at room temperature and thereafter for 24 hr at 4°C. Otherwise the same procedure as described above was used. The difference between the fibrinogen concentrations recorded for the protamine-containing and protamine-free dilutions of plasma is an indication of the concentration of fibrinogen or fibrin complexed with FDP [17].

Platelet counts. The platelet number in blood was determined in only a few animals at the time for the tumour cell injection. Blood was obtained by disposable plastic micropippettes

from a clean cut of the tail-tip. Kristenson's method [18] for staining and counting (number per mm^3 of blood) was used.

Metastasis experiments

In CBA mice the syngeneic sarcoma MCG1-SS [19] was used and in C57 mice the melanoma B16 obtained in 1966 from the Jackson Laboratories (Bar Harbour, Main, U.S.A.) with littermate pairs of the inbred mouse strain. The tumour-cell suspensions were prepared enzymatically with trypsin and DNase [20]. To obtain monodisperse suspensions with maximal viability, the centrifuged cells were resuspended in Parker 199 medium containing 5% syngeneic serum [21]. Cell viability assessed with Trypan blue was above 90% in all suspensions. The cells were injected into a tail vein in a volume of 0·1 ml. MCG1-SS was given in a dose of 5×10^4 cells and melanoma B16 in a dose of 10^6 cells.

In each tumour-host system 2 studies were performed with arvin in single and multiple dose treatment.

Single dose treatment. Fifty units of arvin per kg b.w. were given intraperitoneally 1 hr before the tumour cell injection.

Multiple dose treatment. As pretreatment 6 hr before tumour cell injection 50 units/kg were given intraperitoneally and 4 hr later the same dose intravenously. As continued treatment intraperitoneal doses of 50 units/kg were given 2 hr after the tumour cell injection, 100 units/kg 4 hr later, followed by 3 more doses of 100 units/kg with a 12 hr interval. Thus the last dose was given 48 hr after institution of therapy. To exclude significant bleeding and anaemia in the experimental animals the haemoglobin concentration in blood was determined 24 hr before therapy and 24 hr after its termination. The Merckotest method (E. Merck AG, Darmstadt, W. Germany) was used.

The number of animals in each experiment is shown in Tables 5–7.

The observation period was 15 days for CBA mice, 23 days for C57 mice. A complete autopsy was performed on each mouse, gross metastases

Table 1. *Coagulation parameters in CBA mice given* 50 *units arvin per kg b.w. intraperitoneally. Mice in italics received* 5×10^4 *MCG*1*-SS cells* 1 *hr after arvin injection*

Mouse	Time after treatment (hr)	Whole blood clotting time (min^{sec})	Thrombin clotting time (sec)	Thrombin clotting time, 1 : 1 with 100 mg% fibrinogen (sec)	Fibrinogen mg%	Fibrinogen (mg%) with 10 mg% protamine-sulphate
1	1	>20	>120	>120	0	23
2	1	>20	>120	27	0	26
3	1	>20	>120	>120	0	—
4	1	>20	>120	>120	0	—
Control	1	3^{35}	20	18	160	185
5	4	17^{00}	>120	45	0	53
6	4	>20	>120	38	0	20
7	4	>20	>120	26	0	—
8	4	>20	>120	18	0	—
Control	4	2^{20}	25	20	166	170
9	8	5^{58}	>120	32	15	31
10	8	6^{30}	>120	38	17	32
11	8	4^{20}	>120	34	26	—
12	8	5^{10}	>120	33	26	—
Control	8	4^{20}	26	22	145	135
13	12	2^{30}	35	22	44	48
14	12	2^{35}	30	24	46	64
15	12	3^{43}	48	19	34	—
16	12	2^{40}	33	21	59	—
Control	12	2^{34}	18	18	195	205
17	24	3^{00}	40	22	52	75
18	24	3^{51}	41	20	43	48
19	24	2^{37}	40	17	62	—
Control	24	3^{50}	20	18	174	164
20	48	3^{15}	22	18	97	115
21	48	2^{20}	20	15	95	115
22	48	2^{43}	21	17	102	—
23	48	3^{00}	20	15	177	—

Table 2. Coagulation parameters in C57 BL/6J mice given 50 units of arvin per kg b.w. intraperitoneally

Mouse	Time after treatment (hr)	Whole blood clotting time (min^{sec})	Thrombin clotting time (sec)	Thrombin clotting time, 1 : 1 with fibrinogen 100 mg%	Fibrinogen mg%	Fibrinogen with 10 mg% protamine-sulphate
1	1	>20	>120	52	0	7
2	1	>20	>120	58	0	17
3	1	>20	>120	56	0	—
4	1	>20	>120	54	0	—
Control	1	2^{20}	20	17	107	110
5	8	3^{17}	104	17	6	12
6	8	3^{23}	48	18	26	—
Control	8	2^{30}	21	17	127	128
7	12	3^{37}	26	23	43	51
8	12	2^{15}	24	18	38	31
Control	12	2^{31}	22	17	112	120
9	24	2^{48}	26	18	29	36
10	24	1^{56}	24	17	52	—
Control	24	2^{42}	18	17	150	162
11	48	3^{20}	18	17	123	—
12	48	2^{36}	17	18	108	—
13	72	4^{04}	20	18	105	—
14	72	2^{19}	19	17	96	—

Table 3. Coagulation parameters in CBA mice given multiple arvin doses (intraperitoneally 50 units/kg at 0 and 8 hr, 100 units/kg at 12, 24, 36 and 48 hr; intravenously 50 units/kg at 4 hr). Mice in italics were given 5×10^4 MCG1-SS cells i.v. at 6 hr

Mouse	Time after beginning of treatment (hr)	Whole blood clotting time (min^{sec})	Thrombin clotting time (sec)	Thrombin clotting time, 1 : 1 with fibrinogen 100 mg% (sec)	Fibrinogen mg%	Fibrinogen with 10 mg% protamine-sulphate
1	6	>20	>120	>120	0	32
2	6	>20	>120	25	0	26
3	6	>20	>120	—	0	—
4	6	>20	>120	—	5	—
Control	6	3^{15}	18	17	100	109
5	8	>20	>120	22	0	8
6	8	>20	>120	26	10	13
7	8	>20	—	—	9	—
8	8	>20	—	—	9	—
Control	8	2^{00}	18	18	101	104
9	12	7^{07}	>120	22	0	33
10	12	>20	>120	24	0	10
Control	12	2^{39}	18	15	250	240
11	24	6^{05}	>120	>120	0	39
12	24	5^{05}	>120	69	21	—
13	24	5^{44}	>120	—	27	27
14	24	5^{30}	>120	—	22	32
Control	24	2^{49}	—	—	160	185
15	25	>20	>120	>120	0	9
16	25	>20	>120	>120	0	20
17	36	5^{12}	—	—	24	34
18	36	4^{15}	—	—	23	27
19	48	8^{48}	36	24	0	9
20	48	7^{30}	22	20	33	—
21	56	3^{52}	29	24	21	41
22	56	3^{58}	28	20	58	—
23	72	3^{40}	21	20	22	55
24	72	3^{15}	20	18	32	64
25	96	2^{25}	22	20	52	64
26	96	4^{16}	23	20	90	—
Control	96	2^{40}	19	19	174	164
Control	96	2^{49}	18	16	195	205

Table 4. Coagulation parameters in C57 BL/6J mice given multiple arvin doses (intraperitoneally 50 units/kg at 0 and 8 hr, 100 units/kg at 12, 24, 36 and 48 hr; intravenously 50 units/kg at 4 hr). Mice in italics received 10^6 B16 cells i.v. at 6 hr

Mouse	Time after beginning of treatment (hr)	Whole blood clotting time (min^{sec})	Thrombin clotting time (sec)	Thrombin clotting time, 1 : 1 with fibrinogen 10 mg% (sec)	Fibrinogen mg%	Fibrinogen with 10 mg% protamine-sulphate
1	6	>20	>120	111	0	26
2	6	>20	>120	23	0	23
3	6	>20	>120	—	9	—
4	6	>20	>120	—	11	—
Control	6	3^{56}	19	18	131	137
5	8	>20	—	—	14	22
6	8	>20	—	—	13	36
7	8	>20	—	—	5	—
8	12	>20	92	26	13	31
9	12	8^{54}	54	21	4	10
10	12	8^{23}	—	—	2	10
11	12	>20	—	—	0	—
Control	12	3^{15}	18	17	134	144
12	24	5^{01}	27	20	10	20
13	24	4^{56}	33	22	16	21
14	24	7^{00}	—	—	16	41
15	24	4^{30}	—	—	23	—
Control	24	2^{52}	20	19	100	110
16	36	8^{20}	—	—	13	25
17	36	5^{30}	—	—	13	—
18	48	7^{10}	—	—	17	35
19	48	5^{20}	—	—	15	65
20	48	7^{30}	—	—	6	—
21	48	8^{30}	—	—	11	—
22	54	>20	—	—	17	54
23	54	>20	—	—	11	—
24	54	>20	—	—	17	—
25	56	>20	—	—	9	—
26	56	6^{40}	—	—	24	41
27	56	>20	—	—	17	54
28	56	>20	—	—	5	—
29	72	9^{18}	27	20	12	24
30	72	5^{09}	—	—	26	36
31	96	2^{11}	22	20	70	85
32	96	2^{26}	19	19	100	115

being recorded. In C57 mice the lungs were crowded with confluent metastases, so the lung weights were used to quantify metastases as before [5].

The C57 livers, like the lungs and livers of CBA mice were sectioned for histological examination [22]. Metastases were recorded planimetrically and their total volume in percentage of organ volume, mean volume in mm^3 and number per cm^3 tissue were calculated [23]. These parameters were compared by the two-sample rank test. Incidences of metastases were compared by the Chi-square test and mean numbers of extrapulmonary metastases as described before [5]. Differences with $p < 0{\cdot}05$ were regarded as significant.

RESULTS

Coagulation studies

Only studies with those arvin doses which were used in the metastasis experiments are reported here. It is hoped that studies performed and under way with other dose levels will be the subject of a separate report.

The results are given in Tables 1–4. The sex of the mice is not included, since there was no discernible difference in coagulation parameters between sexes, nor in the susceptibility to Arvin, as long as it was given on a *pro* weight basis.

The *single dose treatment* caused few problems. An intraperitoneal dose of 50 units/kg body

Table 5. Metastases to lungs and liver in CBA mice given 5×10^4 MCG1-SS cells i.v.

Group	No. of animals	To lungs: Incidence	To lungs: Total volume in % of organ tissue	To lungs: Mean volume (mm^3)	To lungs: No. per cm^3 tissue	To liver: Incidence	To liver: Total volume in % of organ tissue	To liver: Mean volume (mm^3)	To liver: No. per cm^3 tissue
Single dose treatment									
Controls	12	12/12	4·4	0·47	95	7/12	0·5	0·41	12
Arvin	12	11/12	2·9	0·32	90	6/12	3·9†	1·10†	36†
Multiple dose treatment									
Controls	12	12/12	4·2	0·53	80	6/12	0·5	0·62	9
Arvin	11*	10/11	7·3	0·58	128	8/11	0·4	0·57	8

*One animal died of unknown reason before termination of the experiment.

†Significant difference from controls ($p < 0·05$).

Table 6. *Gross extrapulmonary metastases in CBA mice given* 5×10^4 *MCG1-SS cells i.v. The incidence for various organs are given. If more than one tumour per organ in an animal, the total number of takes is given within brackets*

	Experimental groups			
	Single-dose treatment		Multiple-dose treatment	
	Controls	Arvin	Controls	Arvin
Total incidence	12/12	11/12	12/12	9/11
Mean number	5·3	6·5	4·0	5·6
Site of takes:				
subcutis	10(21)	7(19)	7(12)	6(9)
skeletal muscle	6(7)	8(14)	8(11)	8(15)
kidneys	6(11)	1	4	2(4)
adrenals	3(4)	3	2	4(5)
ovaries	1	3(4)	2	0
uterine body	1	—	0	1
retroperitoneal fat	2	8(12)	8(11)	6(12)
mesentery	2	6(7)	2(3)	7(8)
mediastinum	1	6(8)	0	2
lymph nodes	6(10)	6(10)	1(2)	4
salivary glands	2	0	0	0

Table 7. *Metastases in C57 BL/6J mice given* 10^6 *melanoma B16 cells i.v. The incidence of gross metastases in various organs are given. If more than one tumour per organ in an animal, the total number of takes is given within brackets*

	Experimental groups			
	Single-dose treatment		Multiple-dose treatment	
	Controls	Arvin	Controls	Arvin
No. of animals	14	14	11*	12
To lungs, incidence	14/14	14/14	11/11	12/12
To lungs, mean organ weight in g (± SD)	0·707(± 0·233)	0·620(± 0·253)	0·431(± 0·203)	0·354(± 0·127)
To other organs:				
Total-incidence	7/14	12/14†	7/11	7/12
Mean number	1·1	2·6†	1·0	1·4
Site of takes:				
subcutis	1	0	1	1
skeletal muscle	1	5	1	3
kidneys	5(10)	7(15)	2	3
adrenals	0	5	3(4)	2(3)
ovary	0	1	0	0
retroperitoneal fat	1	1	2(3)	5(6)
mesentery	1	2(3)	0	2
mediastinum	1	4(5)	0	1
lymph nodes	1	2	0	0

*One animal discarded because of tumour growth at the injection site on the tail.

† Significant difference from controls ($p < 0.05$).

weight achieved incoagulability of blood at the time of tumour cell injection 1 hr later and a prolongation of the thrombin time for 8 hr in both strains. There was a concomitant disappearance of clottable fibrinogen, the level of which rose towards normal values during the subsequent 48 hr. The initial incoagulability seems in part to be due to fibrinogen degradation products (FDP). This can be deduced from the consistently higher fibrinogen titres with protamine sulphate and the prolongation that plasma caused to the thrombin time of fibrinogen [14]. Unfortunately the adopted methods do not permit any precise quantitation of FDP. There is a need to adopt such methods for mice and also to check the normal variation in fibrinogen within murine strains, as has been done with other clotting factors [24]. For, as can be seen in the tables, there was apart from unexpectedly low values considerable variations in the present controls in spite of their inbreeding and similar age.

The *platelet number* was checked before and after arvin (saline) administration in 3 mice of each strain and only at the time for cell injection. There was no trend, the results being:

jections with intervals less than 12 hr caused bleeding intraperitoneally. An extra dose of 50 units/kg could be introduced at 8 hr after the start of treatment, however, without evidence of significant bleeding. This was necessary to prevent the coagulation time from returning to normal before 12 hr. Provided the 12-hr interval was maintained thereafter, dosage increases above 100 units/kg had only marginal effects on the duration of defibrination and incoagulability. With the 100-unit dose, on the other hand, the animals were not totally defibrinated, nor was the blood incoagulable during the whole period of treatment (Table 3 and 4). There was, however, on all occasions a profound hypofibrinogenaemia. The coagulation and thrombin times when tested were also prolonged, but sometimes bordering on normal. After a new injection, as is shown in Table 3 for 25 hr, there was a precipitation of newly formed fibrinogen with the appearance of FDP. Otherwise, the fibrinogen depletion seemed to be the main impediment to coagulation. The treatment was arbitrarily terminated by the 48 hr dose and fibrinogen rose slowly towards normal at 96 hr.

Strain	Treatment	Number ($\times 10^3$) before therapy	Number ($\times 10^3$) 1 hr after therapy
CBA	50 u. arvin/kg	558	472
CBA	50 u. arvin/kg	667	630
CBA	saline	625	525
C57	50 u. arvin/kg	985	1060
C57	50 u. arvin/kg	825	905
C57	saline	1020	1090

When doses for the *multiple dose arvin treatment* were selected problems arose, because mice proved rather resistant to maintenance therapy [cf. 11]. The first dose of 50 units/kg i.p. as above seemed suitable as a start since the animals, apart from the coagulation defect, seemed quite unaffected. It served as a preparation for the same dose given intravenously 4 hr later which also did not cause any overt symptoms. When given without intraperitoneal pretreatment, 50 units/kg i.v. caused mortalities when quickly injected. In all instances it provoked respiratory distress, obviously by pulmonary embolism [25].

Six hours after the beginning of treatment, at the time for the tumour cell injection, the animals were almost totally depleted of clottable fibrinogen. For the continued therapy it was found that repeated intraperitoneal in-

The present data permit no detailed comparison, but there were no overt differences between the two strains of mice. Neither was any extensive comparison made between animals injected with tumour cells and those which were not. But a few samples from tumour-injected animals (Tables 1, 3 and 4) gave no reason to suspect gross differences in this respect.

No gross bleeding complications or significant anaemia developed during the multiple dose treatment. The haemoglobin concentration fell in the metastasis experiment (*vide infra*) by 10·2% in arvin-treated CBA mice (controls 6·2%) and by 11·6% in arvin-treated C57 mice (controls 5·2%).

The *platelet number* fell in some but not all of the mice tested at 6 hr after institution of therapy:

Strain	Treatment	Number ($\times 10^3$) before therapy	Number ($\times 10^3$) after therapy
CBA	Arvin	660	695
CBA	Arvin	760	650
CBA	Saline	625	510
C57	Arvin	1030	765
C57	Arvin	835	753
C57	Saline	950	1100

Metastasis studies

MCG1-SS. The results of the studies with single- and multiple-dose treatment, although performed separately, are shown together in Tables 5 and 6. In animals given a single arvin dose there was no significant alteration of pulmonary metastases or incidence and number of gross extrapulmonary tumour takes. Neither was the incidence of liver metastases altered. The number as well as the average and total volumes of liver metastases were greater, however, in the arvin-treated animals. With multiple dose treatment numerical deviations were seen, but no significant changes were obtained in metastasis parameters. It was remarkable that in each of the two arvin groups, and only here, there was one animal totally free of tumour.

Melanoma B16. In single as well as multiple arvin dose treatment there was some, although far from statistically significant, reduction in lung weights (Table 7). On the other hand, there was a rise in number of gross extrapulmonary takes, although significant only with the single dose treatment. In this group the total incidence of mice with gross extrapulmonary metastases also rose. Liver metastases were seen in only one mouse belonging to the single dose saline-treated control group.

DISCUSSION

With the adopted dosages and modes of arvin administration, it was not possible to keep mice totally defibrinated during any longer period of time. This would otherwise have been the ideal situation for evaluation of the role of fibrin in the establishment of experimental metastases. Instead, the tumour cells were infused into animals in the very dynamic and complex situation soon after diffuse intravascular coagulation (DIC). Arvin has the merit, however, of inducing a less harmful DIC than for instance thrombin [10]. Nevertheless, besides the demonstrated clotting defects an activation of fibrinolysis [7, 8, 25], alteration of thrombocyte functions [26], stimulation of the RES [27] and haemodynamic alterations [11, 28] are among demonstrated components of this situation. Little is known about these effects in mice, but if present they all might affect metastases, singly or in combination [cf. 4].

A state of incoagulability was induced, however, which was maintained for different periods of time. During the multiple-dose treatment the incoagulability, obviously by resynthesis of fibrinogen, partly turned into a state of impaired coagulability. After termination of therapy the effect slowly waned. No evidence was found for a rebound effect in terms of hypercoagulability of hyperfibrinogenaemia. This point was not studied for any longer periods after treatment, but it has not been the case in other species, including man [11, 17, 29].

The influence of arvin as an anticoagulant on experimental metastases may then be compared with that of other anticoagulants. With the tumours presently used the influences of heparin and coumarin have been studied previously [3–5, 22, 30]. To summarize most briefly, heparin in both systems appeared to shunt tumour cells and thereby metastases past the first capillary bed, i.e. the lungs at intravenous injection. Coumarin on the other hand increased B16 metastases in the lungs. MCG1-SS metastases in the lungs were unaltered by coumarin, but the number of gross extrapulmonary metastases rose. Such changes are of course only detectable in systems like the present ones, where metastases develop in several organs.

Melanoma B16 requires too high a transplantation dose for bioassay detection in blood, but it was found by such procedures that irrespective of anticoagulant treatment, viable MCG1-SS cells in the dose used here, 5×10^4 cells, disappear from the circulating blood within 6 hr [31]. The possibility of re-entry of tumour cells into the blood was not entirely ruled out. But most metastases ought to arise from the great majority of cells which are retained in organs during the first few hours

after injection, as demonstrated with both tumours [32, 33].

Even the single dose arvin treatment will then have covered this most important period of primary tumour cell retention. This type of treatment caused an increase of MCG1-SS metastases to the liver and B16 takes in other extrapulmonary organs. A reduction, albeit insignificant, of pulmonary metastases in both systems may point at a certain shunting of tumour cells past the lungs, as for heparin [5, 30]. Fibrin trapping may then be one among other mechanisms for tumour cell retention in this first capillary bed. Facilitated lodgement in other organs in the absence of fibrin cannot be excluded, however. In both cases a similar pattern would have been expected with the multiple dose type of treatment, but this type of treatment caused very little change in metastasis patterns. Only an insignificant reduction of the B16 tumour mass in the lungs persisted and a similar, numerical increase in the mean number of extrapulmonary MCG1-SS metastases. If this discrepancy does not merely reflect the difference in coagulation defect, it could be due to more pronounced systemic effects of multiple dose treatment, counteracting metastasis growth for instance by increased fibrinolysin activity or RES stimulation.

Judged by the seemingly undiminished total metastasis crops in arvin-treated animals, however, the presence of fibrin seems neither to promote the number of cells lodging in tissues primarily, nor to favour their initial growth into metastases. In this respect the results agree with those obtained with the two other types of anticoagulants, militating against the overall importance of fibrin as means for tumour cell retention and support for metastasis growth. These concepts, largely derived from studies in allogeneic tumour-host systems [1, 2, 34, 35], have even in such systems been seriously questioned. Due attention seems not to have been paid to the complexity of changes induced, involving for instance additional effects of anticoagulants [5] and cytotoxicity of fibrinolytic drugs [36]. Similarly, the increasing effect by trauma on metastases represents too complex a situation [cf. 37] to permit the incrimination only of a concomitant rise in plasma fibrinogen [38], which may be an unrelated phenomenon.

Lately, attention has been focused on the role of platelets as a mediator for tumour cell adhesion in vessels [39, 40]. In the present study it was only checked that the quantity of platelets remained essentially undisturbed at the time of tumour cell injection. Arvin reportedly does not affect platelet number of function, which seems to be the reason for the rarity of bleeding complications even in profound defibrination [11, 28, 29, 41]. But FDP may affect platelet functions [26, 42]. Functional tests will have to be performed to clarify whether platelets are involved in the partly diverging effects of different anticoagulants on metastases. Arvin therapy may help to clarify the platelet question further, for instance by combining defibrination with drugs known to alter platelet functions. One has then to be very much aware of the complexity of the situation however. The same is true when arvin, as planned in this laboratory, will be used in combination therapy with other anticoagulants to elucidate their possible additional mechanisms for interaction with metastasis spread.

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Control of Cholesterol Synthesis in Hepatomas:

Absence of Diurnal Rhythm in Hepatomas 7794A and 9618A

JOHN R. SABINE, BRIAN J. HORTON and PETER E. HICKMAN

Department of Animal Physiology, Waite Agricultural Research Institute, University of Adelaide, South Australia, Australia

Abstract—*We have examined the Morris hepatomas 7794A and 9618A for the presence of a diurnal variation in cholesterol synthesis such as is observed in the livers of normal rats.*

Whereas the livers of the tumour-bearing animals showed a 3–5 fold increase in rate of cholesterol synthesis from acetate between midday and midnight, no such increase occurred in the hepatomas. Furthermore, adrenalectomy resulted in a 3-fold increase in cholesterol synthesis (as measured at midday) in the host liver, but not in hepatoma 7794A.

Work by others has indicated that diurnal variations in cholesterol synthesis in normal liver are due to variations in the amount rather than in the specific activity of the rate-controlling enzyme of the cholesterogenic pathway, namely β-hydroxy-β-methylglutaryl co-enzyme A reductase.

Our work has now demonstrated that at least two of the "minimal-deviation" hepatomas exhibit a defect in control over the synthesis and/or degradation of this enzyme, i.e. in addition to the defective "feedback" control of its activity that has been shown in many previous reports.

INTRODUCTION

In 1964 Siperstein and Fagan [1] reported that cholesterol synthesis in the liver tumour was not reduced by cholesterol feeding, a diet known since 1950 [2] to reduce cholesterol synthesis by normal liver. Since then conclusive evidence from a number of laboratories (e.g. [3–5]), including this one [6, 7], has shown that the rate of cholesterol synthesis in the hepatoma, both primary and transplanted, is insensitive to a variety of dietary manipulations known to alter synthesis in the liver, e.g. fasting [3, 6] cholesterol feeding [1, 5, 6] refeeding after fasting [6] and cholestyramine feeding [7].

Several authors [1, 8, 9] have suggested that loss of metabolic regulation exhibited by the tumour may be fundamental in some way to the neoplastic process. This particular control, i.e. over cholesterogenesis, is missing from every hepatoma so far examined [1, 4–7, 10] and furthermore is absent in certain "precancerous" livers, an observation reported first by Siperstein [10, 11] and later by Horton and Sabine [12, 13].

We are now reporting another defect in metabolic control in the transplanted hepatoma. The Morris hepatomas 7794A and 9618A, in Buffalo-strain rats, do not exhibit the diurnal variation in cholesterol synthesis that is a characteristic of normal rat liver [14–16].

Accepted 21 September 1971.

MATERIAL AND METHODS

Animals and tumours

For these experiments we used Buffalo-strain rats bearing the "minimal-deviation" hepatomas 7794A and 9618A. Our original breeding and tumour-bearing animals were imported by air into Australia from the United States,

but subsequently the tumours have been propagated by serial, intramuscular transplantation in this laboratory.

All animals were housed in a windowless but well-ventilated room (21–26°C) and were offered a standard laboratory ration (Charlicks M164 Mouse Cubes) and water *ad libitum*. Lighting was strictly controlled; on at 6 a.m., off at 6 p.m.

Adrenalectomy

Bilateral adrenalectomies were performed under Nembutal anaesthesia, 10 to 15 days prior to the experiment, and the animals subsequently offered both water and NaCl (0·9% in water) to drink. At the time of operation the rats were about 5 to 6 months old, i.e. about 3 months after tumour transplant, and were carrying approximately 4 g of tumour tissue.

Cholesterol synthesis

At either midday or midnight* animals were killed by decapitation and their livers and tumours quickly removed and placed in ice-cold saline (0·9%). The tissues were then sliced with a McIllwain tissue chopper and incubated with either acetate-1-^{14}C or mevalonate-2-^{14}C. Routine procedures for the incubation and subsequent isolation and counting of ^{14}C-labelled cholesterol, as the digitonide, have been described previously [6]. All incubations were in triplicate. Sodium acetate-1-^{14}C and sodium mevalonate-2-^{14}C lactone were purchased from the Radiochemical Centre, Amersham, England.

*Actual experimental times were 1 p.m. and 1 a.m., since under our conditions maximum synthesis occurs about 1 hr after midnight. For convenience, however, we refer to these as midday and midnight.

RESULTS

Table 1 shows the results of experiments examining the diurnal rhythm. It is obvious that whereas the host liver (i.e. the apparently-unaffected liver of the tumour-bearing animal) showed a 3–5 fold difference in rate of cholesterol synthesis from acetate between midday and midnight, no such difference occurred in the tumours. With mevalonate as substrate we observed no consistent, significant difference in rates of cholesterogenesis between midday and midnight for either tissue.

Table 1. *Absence of diurnal rhythm of cholesterogenesis in rat hepatomas*

Expt.	No. of animals		% incorporation of ^{14}C from Acetate-1-^{14}C into cholesterol Midday	Midnight
1	2	Host liver	0·20	1·12
		Hepatoma 9618A	0·52	0·67
2	2	Host liver	0·19	0·60
		Hepatoma 9618A	0·45	0·31
Mean	4	Host liver	0·19 ± 0·02	0·86* ± 0·20
		Hepatoma 9618A	0·49 ± 0·05	0·49† ± 0·11
3	4	Host liver	0·30 ± 0·02	1·19* ± 0·38
		Hepatoma 7794A	0·20 ± 0·02	0·15† ± 0·02

200 mg of tissue slices, from rats killed at midday or midnight, were incubated in 2·0 ml of bicarbonate buffer, pH 7·4, for 2 hr at 37°, with 4 μmoles of acetate-1-^{14}C. Each incubation was done in triplicate, and the number of animals killed at each time is shown. Means are shown ± S.E.M.
*Significantly different from midday values ($P < 0·05$).
†Not significantly different from mid-day values.

Table 2. *Effect of Adrenalectomy on Cholesterogenesis in Morris Hepatoma 7794A*

Treatment		% incorporation of ^{14}C from Acetate-1-^{14}C into cholesterol in Host liver	Hepatoma
Control	(4)	0·25 ± 0·08	0·40 ± 0·05
Adrenalectomised	(4)	0·74 ± 0·14*	0·35 ± 0·10†

Incubation conditions were as in Table 1, with numbers of animals in parentheses. Values given are means ± S.E.M.
*Significantly different from the control values ($P < 0·05$).
†Not significantly different from control values.

Other experiments in this laboratory [17] have shown that adrenalectomy both abolishes the normal diurnal rhythm of liver cholesterogenesis, and causes this synthesis to be maintained at a uniformly high level. Hence we examined the effect of adrenalectomy on cholesterol synthesis by the hepatoma, as measured at midday (Table 2). The results show that adrenalectomy caused a 3-fold stimulation of cholesterol synthesis by the host liver (consistent with our observations on normal animals) but had no effect on cholesterol synthesis by hepatoma 7794A.

DISCUSSION

In seeking to understand the loss of control of cholesterol synthesis in liver tumours we are hampered by lack of precise information as to how this control operates in the normal liver. It is generally accepted [18] that under physiological conditions the primary, though possibly not the sole, point of control in the biosynthetic pathway leading to cholesterol is the enzyme, β-hydroxy-β-methylglutaryl co-enzyme A reductase. In the case of both cholesterol- and cholestyramine-feeding, and perhaps also in the case of short-term fasting, variations in hepatic cholesterol synthesis are believed to occur because of variations in the amount present of an inhibitor of enzyme activity [19]. The precise nature of any inhibitor(s) is as yet unknown, but may relate mainly to absorption of cholesterol from the digestive tract [20]. On the other hand there is strong evidence [14, 21] to suggest that differences in cholesterogenesis between day and night occur primarily because of different amounts of enzyme protein present.

This then is the major significance of the present work. In all previous reports control of cholesterogenesis in hepatomas has failed in situations where an enzyme inhibitor is thought to be involved, whereas in the experiments reported here normal control is almost certainly due to changes in the rate of enzyme synthesis and/or degradation and still the hepatoma was not influenced. Furthermore, our recent work [17] has clearly indicated that one or more adrenal hormones are of prime importance in the normal diurnal rhythm of liver cholesterogenesis, and now we have shown that cholesterol synthesis in the hepatoma is insensitive to the removal of the adrenal glands.

Two hypotheses have been proposed specifically to explain the loss of control of cholesterogenesis in the hepatoma, namely Siperstein and Fagan's [1] of an altered rate-controlling enzyme and Sabine's [6, 7] of altered permeability of the tumour cell to regulatory substances. In the case of the diurnal rhythm of cholesterogenesis in the liver we are unable so far to determine whether this is primarily a phenomenon of control over enzyme synthesis or over enzyme degradation. If degradation is of greater importance in the normal cell, then either of the above hypotheses could be invoked to explain the abnormal behaviour of the tumour cell. But if control of the diurnal rhythm is primarily over enzyme synthesis, then an alteration in the enzyme itself would not explain these results.

Acknowledgements—We gratefully acknowledge the gift of our original tumour-bearing animals from Dr. Harold Morris (Howard University, Washington, D.C., U.S.A.) and the valuable technical assistance of Mrs. Jeanette Horton, Miss Marian Wilson and Mr. Robert Inns. The work was supported, in part, by the Anti-Cancer Foundation of the University of Adelaide.

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NOTE ADDED IN PROOF

Since preparing and submitting this paper we have seen the abstract by Goldfarb and Pitot [22] in which they reported that two hepatomas, 5123C and 7800, show a diurnal variation in activity of β-hydroxy-β-methylglutaryl co-enzyme A reductase, although they lack 'feedback' control of cholesterol synthesis.

We have now examined hepatoma 5123C, and have confirmed that this does show a difference in cholesterol synthesis between midday and midnight ($0{\cdot}25 \pm 0{\cdot}07$ and $0{\cdot}48 \pm 0{\cdot}08\%$ recovery of ^{14}C from acetate-1-^{14}C) comparable to that shown by the liver ($0{\cdot}22 \pm 0{\cdot}03$ and $0{\cdot}64 \pm 0{\cdot}07$). Thus it now appears that not all hepatomas display a defective diurnal rhythm of cholesterogenesis.

If, as seems most likely, normal rhythm is due to the presence of varying amounts of HMG CoA reductase then control over the synthesis and/or degradation of this enzyme in hepatomas shows a pattern similar to that found in other 'enzyme induction' studies in hepatomas (e.g. [23]), i.e. defective in some hepatomas, normal in others.

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Isolation of Prolactin from the "Granular Fraction" of Pituitary-Tumour Transplants:

Improved Method for the Isolation of Mouse and Rat Prolactin for Radioimmunoassay

H. G. KWA, A. A. VERSTRAETEN and M. G. M. SCHEIJDE-BAKKER

Biological Department, Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract—*An improved isolation procedure for rat and mouse prolactin from the "granular fraction" of prolactin producing pituitary tumour transplants is described.*

Proteolytic breakdown of rodent prolactin—by enzymes present in the tumour transplants—often results in conversion of the hormone into electrophoretic components with completely different Rf-values as compared to the intact hormone. This conversion can be diminished when PTI (pancreatic trypsin inhibitor) is used during the isolation procedure. The improvement is seen in: (a) a reduction of the number of N-terminal amino acids, (b) an increased maximal binding—when compared to preparations isolated without PTI—in a ^{125}I-radioimmunoassay and (c) an increased biological activity.

Conversion of rodent prolactin often results in polypeptides with an electrophoretic mobility characteristic of rodent growth hormone, but lacking any growth hormone bioactivity. This finding stresses the relative value of employing electrophoretic migration of pituitary homogenates as the sole basis for estimation of the growth hormone and prolactin content of the pituitary gland.

INTRODUCTION

PROLACTIN is the most effective hormonal agent; it induces mammary tumours in mice of low cancer strains, even in the absence of the milk agent [1, 2]. To analyse the observed differences [3] in the regulation of prolactin secretion between rats and mice, and in view of the incomplete immunological cross-reactivity of rat and mouse prolactin [4], it appeared mandatory to have both rodent prolactins available. The isolation of mouse prolactin from the "granular" fraction of prolactin producing pituitary tumours—essentially using the isolation procedure as described for rats [4]—and a radioimmunoassay based upon this protein has been briefly reported [5]. Difficulties in obtaining reproducible adequate yields withheld us from publishing the experimental details. Bioassay data on mouse prolactin obtained in the occasional experiments with satisfactory yields were in agreement with those obtained with rat prolactin preparations [4]. However, like in the rat, the mouse prolactin was fairly active in some bioassay systems and showed a very low activity in other ones [6].

The present report describes a modified isolation procedure that includes reduction of proteolytic activity, which can almost completely convert mouse prolactin into electrophoretic components with completely different Rf-values as compared to the mouse prolactin present in the starting material.

Accepted 2 August 1971.

MATERIAL AND METHODS

Animals

Male (C57BL × DBA)F_1 mice were orchidectomized at the age of 8 to 10 weeks. The animals were kept in macrolon cages of 18 cm × 11 cm × 13 cm, 3 animals to a cage. The diet consisted of commercial food pellets (Hope Farms, Woerden) and oestrogenized tap water *ad libitum* in the doses described below.

Induction of primary pituitary tumours

At intervals of 6 weeks about 10 mice were put on a regimen of 2 mg oestrone/l. drinking water. Primary tumours of 30 to 100 mg were detected between 300 and 400 days after initiation of the oestrone treatment by the observation of signs of disturbed sense of equilibrium.

Transplantation of pituitary-tumour tissue

Up to 10 pieces of tumour tissue of approximately 1 mg, either from the primary tumours or from subsequent transplants, were rinsed free from excess blood in isotonic buffered sucrose (pH 7·4) and introduced intramuscularly halfway between the knee and ankle joint with the aid of a troicard, through a small incision in the skin near the knee joint. The transplant bearing animals were maintained on an oestrone regimen of 0·5 mg/l., which was sufficient to induce growth of the transplant, but leaved the *in situ* pituitary gland unaffected for up to 250 days, when most of the transplants had reached the size of 1 to 5 g. Three weeks before the transplants were to be used for the isolation experiments the oestrone regimen was stopped and the mice were given tap water *ad libitum.*

Oestrone administration

A stock solution of oestrone in ethanol (200 mg oestrone dissolved in 100 ml ethanol) was kept at 4°C. For the induction of primary tumours 1 ml of the stock solution was added to 1 litre of tap water under constant agitation; for the transplant bearing animals 0·25 ml of the stock solution was added to 1 litre of drinking water.

Preparation of pituitary "brei"

Essentially the same procedure as described for rat pituitary transplants [4] was used, except for the molarity of the sucrose solution: which was 0·3 M in the mouse experiments.

Centrifugation procedure

The centrifugation procedure was essentially as described for the rat transplants [4] except that dextran was obtained from Pharmacia (Uppsala, Sweden), mol wt. 70·000.

Extraction of the sub-cellular fraction

In the experiments, which often resulted in conversion of the hormone into different electrophoretic components with different Rf-values as compared to intact mouse prolactin, the extraction was carried out as described for the rat [4].

The changes introduced recently, which resulted in reproducible and fairly good yields of Mouse-Pituitary Tumour Protein (MPTP) are:

(1) The 40,000 **g** sediment was resuspended in 100 ml 0·025 M borate buffer pH 8·4 and adequate amounts of PTI (Pancreatic Trypsin Inhibitor) [7] were added.

(2) Homogenization was performed in a pressure homogenizer (Baskerville and Lindsay, Manchester, England) according to the method described by Hunter and Commerford [8]. The pressure was maintained at 882 lb/in.^{2} for 15 min and then suddenly released. The homogenate was centrifuged for 20 min at 80,000 **g**, the sediment was discarded. The supernatant was dialysed against 0·025 M borate buffer pH 8·4—which was renewed 4 times—and the dialysate was lyophilized.

Column chromatography and polyacrylamide-gel electrophoresis were carried out as described earlier [4]; in several experiments Sephadex G-100 was used.

Determination of the N-terminal amino acids

The determination of N-terminal amino acids of mouse and rat prolactin was carried out with (1-dimethylamino-naphtalene-5-sulfonylchloride) DANS-Cl according to Gray and Hartley [11]. The chromatographic separation and identification of DANS-derivates was performed on Silica Gel F254 pre-coated plates (Merck, Darmstadt, Germany), layer thickness 0·25 mm [12].

Radioimmunoassay

Rabbit antisera: antiserum 362 was raised in rabbits with the MPTP preparation described earlier [5]. Antisera 466 and 467 were raised in rabbits with the MPTP preparation recently isolated.

Iodination and incubation procedures were carried out as described for rat material [9].

The separation of "bound" and "free" hormone was carried out by the double-antibody technique of Niswender *et al.* [10], with the following modifications:

(1) Rabbit gamma globulin was used instead of normal rabbit serum.

(2) To precipitate the antiserum–antibody complex goat anti-rabbit serum was used at a concentration which maximally precipitated the rabbit gamma globulins present.

After the addition of the goat anti rabbit serum the incubates were allowed to stand for 4 hr at room temperature. Finally the incubation tubes were centrifuged for 10 min at 1000 **g** and supernatant and sediment were separated (which contained "free" and "bound" hormone respectively). The percentage "bound" of a particular reaction mixture was calculated as follows:

$$\text{percentage "bound"} = \frac{\text{cpm "bound"}}{\text{cpm "bound"} + \text{cpm "free"}} \times 100\%$$

RESULTS

Isolation of the "granular" fraction and extraction of the 40,000 **g** *sediment*

Protein distribution was analysed according to electrophoretic mobility on polyacrylamide gels. Figure 1a shows the pattern of the supernatant obtained after deoxycholate treatment of the 40,000 **g** sediment. The amido black stainable material which moves with the indicator front was rather strong; slightly behind this band a fast moving zone was observed which represents prolactin [14–16]. Fig. 1b shows the pattern of the **g** sediment, in presence of an effective amount of PTI. The only band visible after staining with amido black represents prolactin; the buffer front does not contain amido black stainable material any longer.

Column chromatography

Figure 2 shows a representative elution pattern after DEAE-C column chromatography of the deoxycholate extract of the "granular fraction" in the experiments in which no PTI was used. The relative heights of the protein peaks differed from experiment to experiment.

Figure 3 gives the elution pattern of a Sephadex G-100 column. The material applied onto the column had a polyacrylamide gel distribution pattern as shown in Fig. 1a. Figure 4 shows the protein distribution pattern of the main peak eluted from the Sephadex G-100 column. As can be seen almost no material moved to the position where MPTP was expected; instead a new band appeared with an Rf-value of rodent growth hormone. However, no reaction in the tibia test was found.

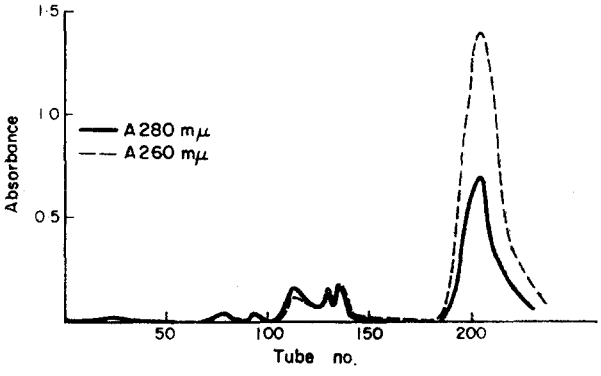

Fig. 2. Elution pattern of deoxycholate extract of the 40,000 × **g** *sediment (not protected by PTI) on DEAE-cellulose. The column (2 × 20 cm) was previously washed and equilibrated with* 0·025 *M borate buffer (pH* 8·4*). Elution was started with the same buffer,* 5 *ml fractions were collected. Starting from fraction* 60 *elution was carried out with a linearly increasing NaCl-gradient from* 0–1·0 *M, in the same buffer.*

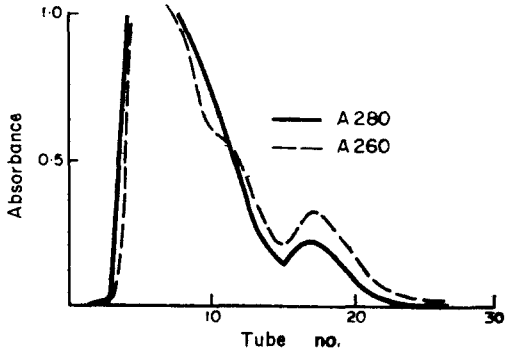

Fig. 3. Elution pattern of deoxycholate extract of the 40,000 × **g** *sediment on a Sephadex G-100 column (2 × 20 cm). Elution was carried out with* 0·083 *M Tris buffer (pH* 8·9*) in the presence of* 3·5 *mM EDTA.*

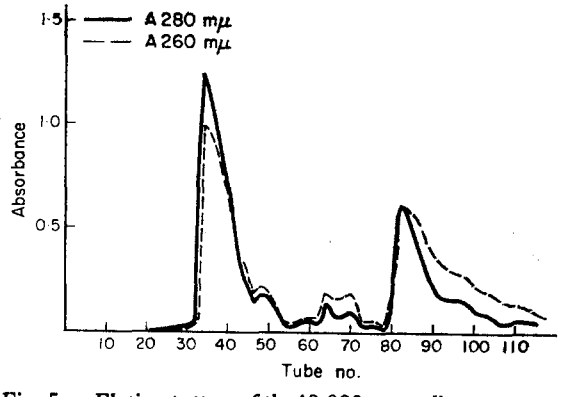

Fig. 5. Elution pattern of the 40,000 × **g** *sediment, protected by PTI, after pressure homogenization. The DEAE-cellulose column was washed and previously equilibrated with* 0·025 *M borate buffer (pH* 8·4*); elution was started with the same buffer. Starting from fraction* 30 *elution was carried out with* 0·15 *M NaCl in the same buffer. From fraction* 70 *onward elution was performed with* 0·30 *M NaCl in the same borate buffer.*

Figure 5 shows the elution pattern from a DEAE-C column of the material (40,000 **g** sediment after pressure homogenization) protected by the addition of PTI. Application of 20 μl samples of the lyophilized peak fraction onto polyacrylamide gels showed a heavily stained zone at the position of MPTP [Fig. 6(1)]. Figure 6(2) shows the improvement by the application of PTI in the isolation of rat prolactin. As can be seen the heavily-stained front band (see [4], Fig. 3c) has completely disappeared.

Determination of the N-terminal amino acids of mouse and rat prolactin

After the application of PTI for the isolation of rat prolactin a dramatic reduction of N-terminal amino acids was observed. In a few experiments only one N-terminal amino acid was found: asp-NH_2.* In some experiments asp-NH_2 and alanine were present in about equal amounts.

The mouse material showed two N-terminal amino acids: phenylalanine and valine present in about equal amounts.

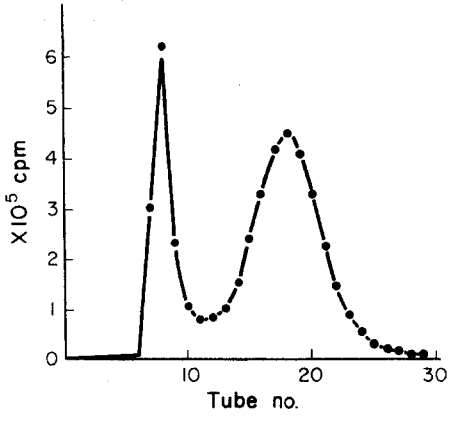

Fig. 7. Elution pattern of a Sephadex G-50 (coarse) column (1 × 10 cm) showing the separation of ^{125}I-labelled MPTP from unreacted $Na^{125}I$. 25 μg MPTP was labelled with ± 1 mC $Na^{125}I$, iodination efficiency ± 30%. Only tube 8 was used. An aliquot from this tube was diluted, with diluent buffer, to 12 ng labelled MPTP per ml.

*In a preliminary pigeon-crop assay—kindly performed by Dr. I. A. Forsyth—the activity of a preparation with a single N-terminal amino acid was roughly estimated to be around 12 i.u./mg (Dr. I. A. Forsyth, personal communication). The earlier preparations which had many N-terminal amino acids were estimated by pigeon-crop assay to have a biological activity of 2–3 i.u./mg [6].

Radioimmunoassay

Labelling of MPTP: The iodination efficiency in a representative labelling experiment was about 30%. The elution pattern from the Sephadex G-50 column (1 × 10 cm) is represented in Fig. 7.

Comparison of antiserum titration curves employing homologous and heterologous antisera: Fig. 8a shows the antiserum titration curves obtained with labelled MPTP and antisera to MPTP and RPT (rat prolactin). Note that the maximal binding in the homologous system is only 40–50%. By adding PTI during the homogenization of pituitary material the maximal binding has increased to 60 to 65%. Figure 8b gives the antiserum titration curves employing labelled RPT as antigen using the two antisera as indicated in Fig. 8a. The RPT used for labelling was protected by PTI during its isolation. It may be stressed here that the maximal binding is 81%—compared to 50–60% obtained with the earlier preparations [9]—in the homologous system, and 73% when the heterologous antiserum was used. The lines do not coincide [F_{coinc} = 45·06, $F_{0.95}(2, 6)$ = 5·14] but may be considered parallel to each other [F_{par} = 0·034, $F_{0.95}(1, 6)$ = 5·99].

Comparison of competitive inhibition curves

Figure 9 represents from left to right the inhibition curves obtained in a radioimmunoassay system employing labelled MPTP and its homologous antiserum by addition of respectively a homogenate of normal mouse pituitaries (open circles) diluted from starting material, which contained 10 mg (w/w) of normal ♂♂ $(C_{57}BL \times DBA)F_1$ pituitary tissue per ml, MPTP in ng/ml (closed circles) and RPT in ng/ml starting from 12×10^2 (triangles),

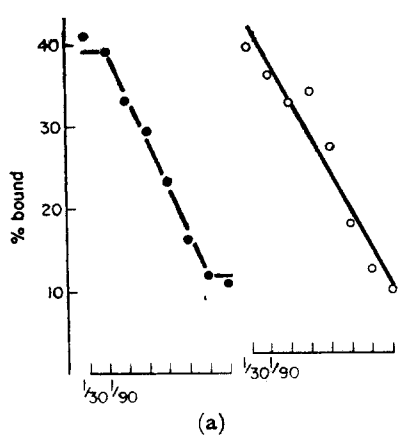

Fig. 8. Antisera titration curves employing homologous andheterologous antisera.

(a) ● *antiserum to MPTP, and labelled MPTP.*
○ *antiserum to RPT, and labelled MPTP.*

(b) ○ *antiserum to MPTP, and labelled RPT.*
● *antiserum to RPT, and labelled RPT.*

Fig. 1. *Polyacrylamide-gel electrophoresis of mouse prolactin.* (*a*) 40,000 × **g** *sediment after deoxycholate treatment.* (*b*) 40,000 × **g** *sediment after pressure homogenization in the presence of PTI* (*pancreatic trypsin inhibitor*). *Electrophoresis for* 50 *min at* 110 *V measured on electrodes,* 5 *mA per tube. Amido Black staining.*

(to face p. 36)

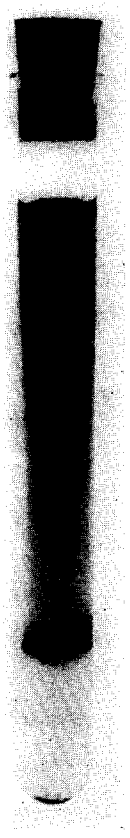

Fig. 4. *Polyacrylamide-gel electrophoresis of the main peak eluted from the Sephadex G-*100 *column (Fig.* 3*). Electrophoresis for* 50 *min at* 110 *V measured on electrodes,* 5 *mA per tube. Amido Black staining.*

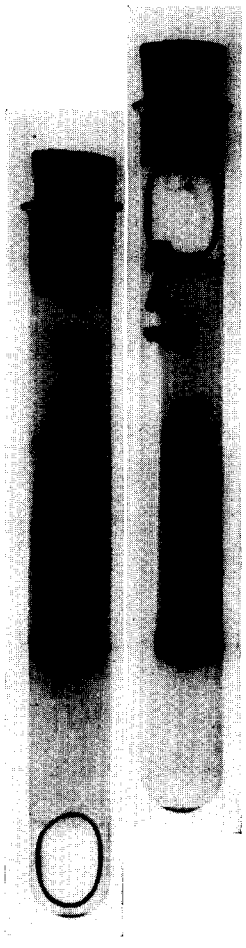

Fig. 6. *Polyacrylamide-gel electrophoresis of rodent prolactin after DEAE-cellulose chromatography.*
(1) *Lyophilized peak fraction from the DEAE-cellulose column (Fig.* 5*).*
(2) *Lyophilized RPT isolated in the presence of PTI.*

12×10^3 (open squares) and 12×10^4 (closed squares) respectively. Homogenates of normal rat pituitaries showed no inhibition at the starting concentration of 10 mg (w/w) pituitary tissue per ml.

The regression lines obtained with MPTP and a homogenate of normal mouse pituitaries run fairly parallel to each other. In contrast the regression lines obtained by inhibition of the binding reaction between labelled MPTP and its antiserum by unlabelled RPT show a distinctly flatter slope [$F_{par} = 23{\cdot}1$, $F_{0{\cdot}95}(2,6) = 5{\cdot}14$].

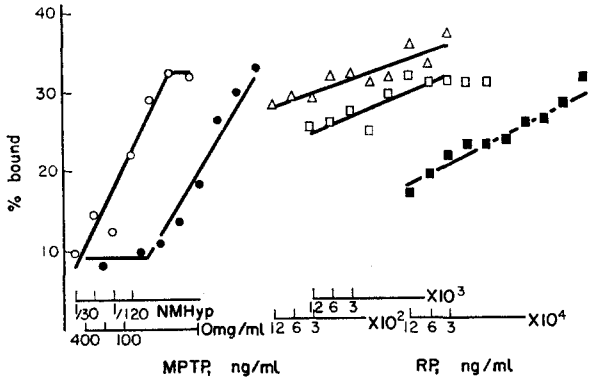

Fig. 9. Inhibition of the building of ^{125}I-labelled MPTP and an antiserum to MPTP by serial dilutions of:

- ○ *A homogenate of normal mouse pituitaries (♂♂ (C57Bl-X-DBA) F_1), starting from 10 mg tissue (w/w) per ml.*
- ● *MPTP, starting from 400 ng/ml.*
- △ *RPT starting from 1200 ng/ml.*
- □ *RPT starting from 12,000 ng/ml.*
- ■ *RPT starting from 120,000 ng/ml.*

DISCUSSION

To our knowledge this is the first report of a successful isolation procedure of mouse prolactin with reproducible good yields. Only after application of PTI early during the isolation procedure was break-down of the hormone prevented. Apparently pituitary tumour transplant material contains an abundance of proteolytic activity. This resulted in the rat in partial degradation of prolactin as was indicated by a heavily stained zone at the position of the indicator front [4], when disc-electrophoresis was performed. In the mouse experiments the proteolytic enzymes often resulted in conversion of the molecules to polypeptides with a completely different protein distribution pattern on polyacrylamide-gels, concomitant with a loss of biological as well as immunological activity. In the rat this proteolytic activity may have been the cause for the finding of 0·1 mole of serine, 0·05 mole of leucine and between 0·01 mole of Asp, Glu, Ala, Val and Thre per prolactin molecule (MW: 23,000) as N-terminal amino acids determined by the DNFB-method [9]. However, apparently the cleaving off of amino acids or peptides from the rat material resulted in enough prolactin molecules, which had a sedimentation coefficient consistent with a molecular weight of 23,000 as described for rat prolactin isolated by Ellis *et al.* [13]. The partially degraded rat prolactin was immunologically still active and could be used for radioimmunoassay giving estimates in unit weights of rat prolactin in biological samples, which did not differ significantly from those obtained by employing the Ellis preparation [9]. The present finding of a single N-terminal amino acid, which is not leucine as reported by Ellis *et al.* [13], but Asp-HN_2 suggests the possibility of a precursor of prolactin.

The results in the mouse material were greatly improved by the application of PTI in the isolation of the hormone; the elution pattern of the "granular fraction" became reproducible and resulted in yields of $\pm 1{\cdot}5$ mg prolactin per gram starting material. However, partial degradation apparently still occurs. Not only that more than one N-terminal amino acid was found, but also the maximal binding of the mouse preparation after labelling with ^{125}I did not attain values comparable to those obtained with labelled RPT. The maximal binding obtained with antiserum to rat as well as to mouse prolactin was around 50% of the labelled mouse prolactin preparation, with neither difference in maximal binding nor slope, but only with a difference in titre. A difference in titre as well as in the maximal binding of around 70% of the labelled rat preparation by the heterologous antiserum was observed, compared to more than 80% by the antiserum to rat prolactin. These findings are in agreement with the reported partial cross-reaction of the two rodent hormones (by spur formation of the two precipitation lines) in the Ouchterlony double-diffusion test [4].

Nevertheless the results of the inhibition experiments indicate the necessity of isolating mouse prolactin and raising an antiserum to it in order to be able to do radioimmunoassays of mouse plasma prolactin levels in physiological conditions. In spite of partial degradation of the mouse prolactin preparation as evidenced by a low maximal binding by the two antisera raised against the two rodent prolactin preparations, it is better—in view of the differences in the slopes of the regression lines of MPTP and RPT—to assay plasma prolactin values in mice with the homologous radioimmunoassay system.

As was shown by Kwa and van der Gugten [17] it is possible to assay quantitatively the increased plasma prolactin levels of isograft bearing ♀♀ mice with a radioimmunoassay system employing labelled RPT, in combination of an antiserum to MPTP and with MPTP as reference preparation. However, with this combination it proved impossible to assay physiological fluctuations of plasma prolactin levels in normal ♀♀ mice.

The experience reported in this paper of conversion of mouse prolactin into Amido Black stainable material, which has an electrophoretic mobility on polyacrylamide-gels characteristic of rodent growth hormone—but which lacks any growth hormone bioactivity—underlines the dangers of employing electrophoretic migration of pituitary homogenates as the sole basis for estimations of growth hormone and prolactin content of the pituitary gland.

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Effect of Pituitary Tumours and Grafts on Plasma Prolactin Levels

H. G. KWA, A. A. VAN DER GUGTEN, M. SALA and F. VERHOFSTAD

Biological Department, Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract—*Using radioimmunoassays the influence on the plasma prolactin levels of oestrone and pituitary isografts in mice and of oestrone, pituitary isografts and chloorpromazine in rats, have been observed in a number of experiments. Suggestive evidence is given for the existence of a Prolactin Releasing Agent in Rats.*

INTRODUCTION

IN VIEW of the extensive use of many highly inbred strains of mice in cancer research the relatively few reports indicate that spontaneous tumours of the anterior pituitary are not common in this species. In rats on the other hand, they are of common occurrence in both sexes of several inbred strains [1].

Experimental induction of pituitary tumours involves a long term derangement of the hormonal balance between the pituitary and its target glands; one of the most effective derangements of the hormonal balance that will result in tumourous growth of the pituitary in mice and rats is long term oestrogen administration. Cramer and Horning [2, 3] were the first to report the finding of pituitary tumours in mice and Zondek [4] and McEuen *et al.* [5] reported the finding of pituitary adenomata in rats after oestrone administration. Most of the literature on oestrogen-induced pituitary tumours has been reviewed by Russfield [1].

Another very effective procedure to obtain pituitary tumours in mice is the isologous transplantation of a normal pituitary gland [6–11]. Since females are more apt to develop tumours than males and ovariectomy of the host will decrease tumour formation, endogenous oestrogen may contribute to tumourous growth of the isograft besides the release from hypothalamic inhibitory factors. In rats no tumourous growth of pituitary isografts have been reported with the exception of a report from Kullander [12] that pituitaries transplanted into the anterior chamber of the eye will form tumours, but only if a fragment of functional ovary is implanted near the pituitary graft. This suggests that in the rat the isograft is dependent on stimulation by rather high oestrogen levels acting directly on the grafted pituitary tissue to become tumourously enlarged, whereas in mice apparently when the pituitary tissue is released from hypothalamic inhibitory factors by grafting away from the sella turcica physiological levels of oestrogens suffice to do so.

We recently reported plasma prolactin estimates in rats with spontaneous pituitary tumours, primary oestrone-induced pituitary tumours or pituitary tumour transplants [13]. In the present report we extend the observations to mice with primary oestrone-induced pituitary tumours and to mice and rats bearing isologous pituitary grafts under the kidney capsule. For comparison with the latter group, observations in rats that were chronically treated with chloorpromazine with or without oestrogen supplementation, are included. The results are discussed and related to differences in prolactin regulatory mechanisms between the two species. The relevance of these differences to differences in effectivity of various experimental procedures designed to induce mammary tumours is stressed. It is suggested that in both species prolonged hyperprolac-

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tinaemia results in mammary cancer; the difference between the two species being that hyperprolactinaemia of long duration is relatively easily attained in mice but difficult to induce in rats.

MATERIAL AND METHODS

Animals

F_1-hybrid mice and rats of the highly-inbred strain R-Amsterdam were used as plasma sample donors for all the experiments reported. Mice were kept in macrolon cages of 17·5 × 11·5 × 12·5 cm and rats in macrolon cages 38 × 25 × 15 cm; two or three animals to a cage, under controlled illumination (14 hr light and 10 hr darkness). The diet consisted of commercial food pellets (Standard Laboratory Diet, Hope Farms, Woerden, Holland) and tap water *ad libitum*, unless otherwise stated.

Experiments in mice

(*a*) Five male $(C_{57}BL \times DBA_f)F_1$ mice were orchidectomized at the age of 8 weeks. Starting from the day of operation (day 0) the mice received oestrone in their drinking water (2 mg/l.). Blood samples for prolactin estimation were collected on days 24, 80, 120, 150, 178, 210, 259, 320, 350, 378 and 406.

(*b*) Six female $(C_{57}BL \times CBA)F_1$ mice received a pituitary isograft under their left kidney capsule at the age of 8–10 weeks (day of operation = day 0). Blood samples were collected on days 150, 195, 273 and 382.

(*c*) Five males $(C_{57}BL \times CBA)F_1$ mice were orchidectomized at the age of 8–10 weeks. An isologous pituitary gland was grafted under their left kidney capsule at the same sitting. Starting from the day of operation (day 0) the mice received oestrone in their drinking water (0·25 mg/l.). Blood samples were collected on day 195 only.

Experiments in rats

Fifteen female rats and fifteen male rats were castrated at the age of 12 to 14 weeks. Ten animals of each group received a pituitary isograft under their left kidney capsule at the same sitting. Five of these isograft bearing animals of each sex received oestrone in their drinking water (2 mg/l.), starting from the day of operation (day 0). Blood samples were taken on days 8, 14, 22, 25, 32, 50, 95, 116, 196, 330, 378 and the day of autopsy (day 506). On the day of autopsy the *in situ* pituitary glands have been weighed, homogenized in phosphate buffered saline and assayed for prolactin concentration. These animals are represented in the female groups a, b and e, and the male groups h, i and l.

Twenty female and twenty male rats at the age of 12 to 14 weeks have been distributed over 4 female groups (c, d, f and g) and 4 male groups (j, k, m and n); the rats of 3 groups of both sexes were castrated (c, f, g and j, m, n). Starting on the day of operation (day 0) the spayed and the orchidectomized rats received either chloorpromazine, 375 mg/l. (c and j), or oestrone, 2 mg/l. (f and m), or a combination of both drugs, 375 mg chlp + 2 mg oestrone/l. (g and n) dissolved in their drinking water. The two groups of intact rats (d and k) received only chloorpromazine, 375 mg/l. drinking water. Blood samples have been taken on days 7, 14, 49, 64, 88, 143, 165, 176, 301, 349, 405 and on the day of autopsy (day 405 for groups c, d, g, j, and k, day 476 for groups f and m), when the *in situ* pituitary glands have been weighed, homogenized in phosphate buffered saline and assayed for prolactin concentration.

Five male rats (group o) were orchidectomized at the age of 12 weeks and received a pituitary isograft under their left kidney capsule at the same sitting. Starting from the day of operation (day 0), a 2 mg oestrone–cholesterol pellet (1 : 9 w/w) was implanted subcutaneously in each rat fortnightly and the rats received oestrone in their drinking water (2 mg/l.). Blood samples were taken only at day 195 and the day of autopsy (day 315), when the pituitary glands *in situ* were weighed homogenized in P.B.S. and assayed for prolactin concentration, and the isografts were inspected for outgrowth.

The results of the experiments in rats are presented in the following order.

Experiments with spayed female rats without oestrone supplementation.

(a) Five spayed female rats.

(b) Five spayed female rats bearing a pituitary isograft under their renal capsule.

(c) Five spayed female rats treated with 375 mg chloorpromazine/l. drinking water.

Experiments with normal female rats, or with spayed female rats with oestrone supplementation.

(d) Five intact female rats treated with 375 mg chloorpromazine/l. drinking water.

(e) Five spayed female rats bearing a pituitary isograft under their renal capsule and treated with 2 mg oestrone/l. drinking water.

(f) Five spayed female rats treated with 2 mg oestrone/l. drinking water.

(g) Five spayed female rats treated with both 375 mg chloorpromazine and 2 mg oestrone/l. drinking water.

Experiments in orchidectomized rats without oestrone supplementation.

(h) Five orchidectomized rats.

(i) Five orchidectomized rats bearing a pituitary isograft under their renal capsule.

(j) Five orchidectomized rats treated with 375 mg chloorpromazine/l. drinking water.

Experiments with intact male rats treated with chloorpromazine and with orchidectomized rats with oestrone supplementation.

(k) Five intact male rats treated with 375 mg chloorpromazine/l. drinking water.

(l) Five orchidectomized rats bearing a pituitary isograft under their renal capsule and treated with 2 mg oestrone/l. drinking water.

(m) Five orchidectomized rats treated with 2 mg oestrone/l. drinking water.

(n) Five orchidectomized rats treated with both 375 mg chloorpromazine and 2 mg oestrone/l. drinking water.

Effect of fortnightly oestrone-pellet implantation in orchidectomized rats bearing a pituitary isograft.

(o) Five orchidectomized rats bearing a pituitary isograft under their renal capsule and treated with both 2 mg oestrone/l. drinking water and fortnightly a 2 mg oestrone–cholesterol pellet.

Oestrone administration

Oestrone–cholesterol pellets were prepared and implanted as described in detail elsewhere [13].

Oestrone dissolved in drinking water was prepared twice weekly by adding appropriate amounts of a stock solution (200 mg oestrone dissolved in 100 ml ethanol) to 1 l. tap water. The oestrone dose in drinking water sufficient to result in vaginal cornification in spayed animals, is 0·5 mg/l. for rats and is 0·125 mg/l. for mice.

Collection of blood samples

Blood samples were collected between 11·00 and 12·00 a.m. from the occular venous plexus. The method for rats has been described in detail elsewhere [14]. For mice 100 μ pipettes with bent tips were employed, connected to a piece of plastic tube with mouth piece. The pipette was wetted with a heparine solution (2% heparine in phosphate buffered saline) by sucking the solution ± 1 cm past the constriction and expelling it, repeated 3 times. The bent tip was then introduced into the occular venous plexus and blood drawn into the pipette up to the constriction by gentle suction; the tip withdrawn (bleeding is stopped by pressing the closed eye for a few seconds) and the content is expelled into a small polystyrene tube. The heparinized buffered saline solution is sucked up to the constriction and expelled into the polystyrene tube twice. The tube is centrifuged for 10 min at 500 × **g**; the supernatant is pipetted into another polystyrene tube and either frozen and stored at −20°C or immediately used for the assay.

Radioimmunoassay procedures

The mouse, rat and bovine prolactin preparations, rabbit antisera, iodination procedure, incubation of reaction mixtures have been described elsewhere [14–16]. Separation of bound and free hormone was carried out by the double-antibody technique using an antiserum to rabbit gamma-globulin produced in a goat.

RESULTS IN MICE

Primary oestrone-induced pituitary tumours in mice

Experiment a. The assay results of following the prolactin levels in the circulation of orchidectomized mice kept on a regimen of oestrone dissolved in their drinking water (2 mg/ml) are summarized in Fig. 1. Plasma levels in

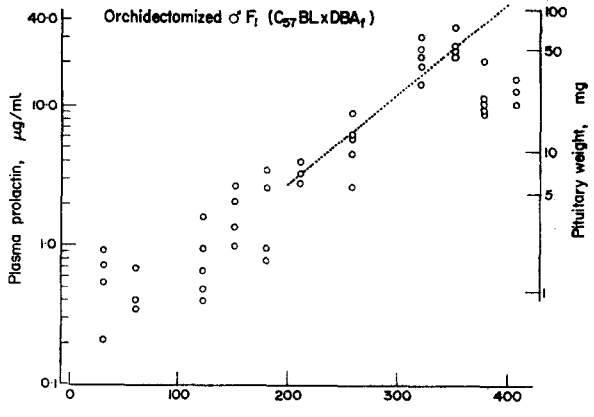

Fig. 1. Prolactin levels in plasma of orchidectomized mice (experiment a) treated with 2 mg oestrone/l. drinking water. Each circle represents the plasma prolactin concentration of one animal. The dotted line represents the mean pituitary-weight curve in ($C_{57}BL \times DBA_2)F_1$ mice. See text. The prolactin values are expressed in µg equivalents of the reference preparation per ml of plasma, plotted logarithmically on the left vertical axis. The pituitary weights, in mg, are plotted logarithmically on the right vertical axis. The days after initiation of treatment are plotted linearly on the horizontal axis.

untreated orchidectomized mice of this strain are below the sensitivity of the assay (45 mg/ml). The oestrone administration increased the plasma levels significantly. Plasma levels were in an easily measurable range by day 24. The assay results of day 80 suggest a slight decrease to have occurred between day 24 and 80; the difference, however, is not statistically valid. By day 120 the plasma levels have recovered and appear to increase from that period onward until day 350 in a geometrical fashion. The prolactin values being plotted on the left vertical axis in a logarithmic scale and the days of oestrone treatment horizontally in a linear scale, the geometrical increase of the plasma prolactin values results in points, which cluster along a straight line.

The weights of the pituitary tumours induced in various strains of mice by oestrone in drinking water (2 mg/l.), when plotted semilogarithmically against the duration of treatment cluster along straight lines, the angles of these lines are characteristic for the strain of mice [17]. In orchidectomized $(C_{57}BL \times DBA_f)F_1$ mice the straight line representing the mean pituitary tumour weights extended to approximately day 450 of the treatment, when the tumours reached an average weight of around 200 mg. The dotted line drawn in Fig. 1 represents the mean pituitary weight curve obtained in the earlier experiment (the corresponding pituitary weights are represented on the right vertical bar). The remarkably good fit of the mean weight curve and the cluster of plasma prolactin values obtained in the present experiment indicates that the prolactin output per unit weight of pituitary tissue remained fairly constant up to day 350 of oestrone treatment.

After day 350, however, there is a dichotomy between the further increase of pituitary tumour weights (which keep on increasing exponentially at the same rate), and the plasma prolactin values (which show a statistically significant fall between day 350 and days 378 and 406).

Isograft-bearing female mice

Experiment b. The exponential increase in plasma prolactin levels reported earlier [18] in isograft-bearing female mice in the period between 6 and 18 months after isografting were based on results obtained with a radioimmunoassay system employing a protein isolated from the "granular fraction" of oestrogen-induced prolactin-producing in mice [15]. Since this preparation has never been compared with a prolactin preparation isolated from normal mouse pituitaries, it was decided to check the earlier results. However, no mouse prolactin has been isolated from normal mouse pituitaries up to now. Therefore it was decided to use for this purpose normal rat prolactin made available by Dr. Ellis [14]. The assay system consisted of labelled normal rat prolactin employed in combination with the antiserum raised to mouse (tumour) prolactin and the mouse preparation was also employed as reference preparation.

The results of this experiment are represented by open circles in Fig. 2. The dashed line represents the mean regression line based on the earlier assay results [employing the mouse (tumour) prolactin as labelled preparation]. As can be seen the results of the present experiment employing normal (rat) prolactin as labelled hormone, cluster nicely along the mean regression line based on assay results obtained earlier, when labelled mouse (tumour) prolactin was used.

The dotted line in Fig. 2 represents the mean weight increase of the isograft in intact female mice of this F_1-hybrid combination as reported by Boot *et al.* [19]. In contrast to the good fit

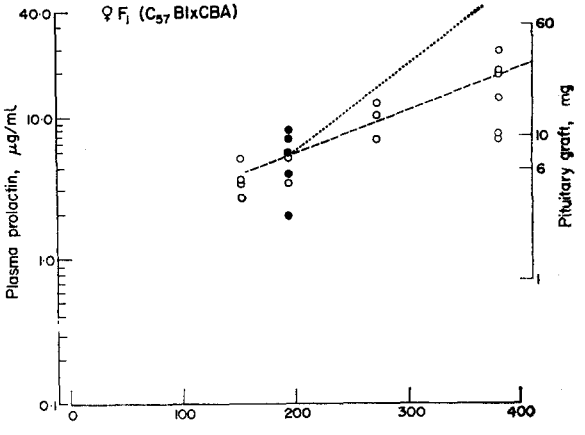

Fig. 2. The plasma prolactin concentrations in female mice bearing a pituitary isograft (open circles, experiment b), and orchidectomized mice bearing an isograft and treated with 0·25 mg oestrone/l. drinking water (closed circles, experiment c). The dashed line represents the mean regression line. The dotted line represents the mean weight increase of the isograft in intact $(C_{57}BL \times CBA)$ F_1 female mice. See text. Further details see Fig. 1.

observed in mice with primary oestrone-induced pituitary tumours between the mean regression lines representing the pituitary weights and the plasma prolactin levels respectively, the mean regression line representing the weights of the isografts is steeper than the one representing the increase in plasma prolactin levels in these animals. Apparently, the prolactin output per unit weight of isografted pituitary tissue is less

than that of the pituitary gland *in situ* under oestrogen stimulation. The straight regression line representing the increase in plasma prolacting levels with time, however, indicates that the output per unit of weight remains remarkably constant.

Isograft-bearing orchidectomized mice

Experiment c. The assay results of plasma values in orchidectomized mice after 195 days on a regimen of 0·25 mg oestrone/l. of drinking water are represented by closed circles in Fig. 2. They indicate that isografts in orchidectomized male mice continuously supplemented with oestrogens in the "physiological dose range" not only grow as well as isografts in female mice, but also contribute to prolactin levels in the circulation comparable to that of isografts in female mice.

Results in rats

The results of plasma prolactin estimations in rats and the *in situ* pituitary weights and prolactin concentrations are summarized in Table 1 (experiments with female rats) and Table 2 (experiments with male rats) respectively. As can be seen the rats in almost all experimental groups show a pronounced dip in their prolactin levels around 50 days after the initiation of treatment. It should be stressed that the experiments with chloorpromazine were started 30 days later than those with pituitary isografts. It rules out that the observed dip does not reflect a real decrease in plasma prolactin levels, but has been caused by a systematic error inherent in assay methods employing a reference preparation, e.g. a "drift" in the prolactin preparation employed as standard in the radioimmunoassays.

Experiments with spayed female rats without oestrone supplementation

Experiments a, b, c. As can be seen from Fig. 3 all three groups of female rats without oestrone supplementation after castration show essentially the same general pattern: in the first period of observation the plasma levels start to fluctuate between mean values of 0·1 and 0·2 μg/ml with exception of the spayed rats bearing pituitary isografts, who exhibit far greater fluctuations in the first 3 weeks of observation, i.e. 0·27 μg/ml at day 7; 0·015 μg/ml at day 14 and 0·15 μg/ml at day 22, dropping to mean values of between 0·01 μg/ml and 0·04 μg/ml around day 50. By day 100 the plasma-prolactin levels have been restored again in all groups to mean values of around

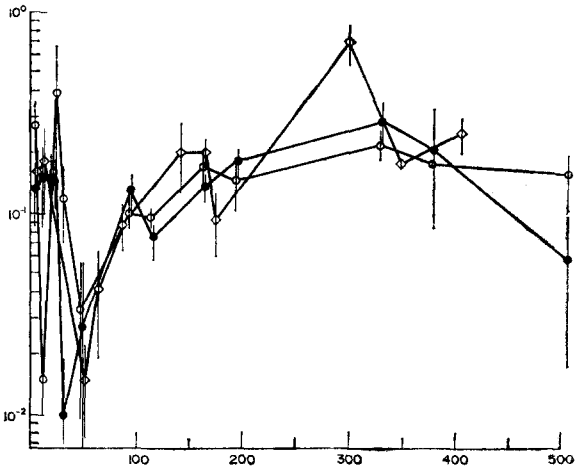

Fig. 3. Prolactin levels in plasma of spayed rats under various experimental conditions. See text. Each dot represents the mean plasma prolactin level of an experimental group of rats. The vertical bars represent the standard deviation. The values are expressed in μg equivalents of the reference preparation per ml of plasma, plotted logarithmically on the vertical axis. The days after initiation of treatment are plotted linearly on the horizontal axis.

(*a*) ● *Spayed rats.*
(*b*) ○ *Spayed rats + pituitary under renal capsule.*
(*c*) ◇ *Spayed rats + 375 mg chloorpromazine/l. drinking water.*

0·1 μg/ml and remain for the next 300–400 days at mean values of between 0·1 μg/ml and 0·3 μg/ml, except for one set of observations in the spayed rats treated with chloorpromazine, who showed a mean plasma value of 0·69 μg/ml on day 301.

The mean weight of the *in situ* pituitary glands on the day of autopsy (day 506 for groups a and b; day 405 for group c) were respectively 13·0 ± 1·4 mg, 12·0 ± 0·8 mg and 15·6 ± 1·4 mg. The difference between groups a and b and between groups a and c is statistically not valid. The difference in weight between the pituitary glands in the spayed rats bearing a pituitary isograft and in the spayed rats treated with chloorpromazine, was statistically significant (b<c, $p<0{\cdot}01$, Student's *t*-test).

The prolactin concentration of the pituitary glands (μg/mg w.w.) did not significantly differ in groups a and b (i.e. 0·322 ± 0·138 and 0·405 ± 0·024) and in groups a and c (c = 5·88 ± 3·43). The latter is probably due to the fact that only two animals of group a had remained. The other animals of this group had died accidentally on day 420. A significant difference has been noticed between groups b and c (b<c $0{\cdot}01<p<0{\cdot}05$, Student's *t*-test).

Experiments with normal female rats, or with spayed female rats with oestrone supplementation

Experiments d, e, f, g. As can be seen from Fig. 4 all four groups of female rats with either endo-

Table 1

Day	sp. ♀♀+P.I. +oestr.	sp. ♀♀+P.I.	sp. ♀♀	sp. ♀♀+oestr.	sp.♀♀+Clpr +oestr.	sp. ♀♀+Clpr	♀♀+Clpr
7	0·047 ± 0·027 $n = 5$	0·272 ± 0·075 $n = 5$	0·131 ± 0·045 $n = 5$	0·198 ± 0·109 $n = 4$	0·141 ± 0·056 $n = 5$	0·158 ± 0·053 $n = 5$	0·269 ± 0·178 $n = 4$
14	0·129 ± 0·118 $n = 4$	0·015 ± 0·005 $n = 5$	0·150 ± 0·066 $n = 4$	0·188 ± 0·072 $n = 4$	0·166 ± 0·087 $n = 5$	0·179 ± 0·081 $n = 4$	0·042 ± 0·027 $n = 5$
22	0·204 ± 0·070 $n = 4$	0·161 ± 0·031 $n = 4$	0·151 ± 0·050 $n = 4$				
25	0·150 ± 0·025 $n = 3$	0·389 ± 0·284 $n = 4$	0·144 ± 0·032 $n = 5$				
32	0·030 ± 0·014 $n = 5$	0·118 ± 0·050 $n = 4$	0·010 ± 0·009 $n = 5$				
50	0·043 ± 0·027 $n = 4$	0·033 ± 0·024 $n = 4$	0·027 ± 0·027 $n = 4$	0·018 ± 0·013 $n = 5$	0·035 ± 0·017 $n = 5$	0·015 ± 0·008 $n = 5$	0·029 ± 0·105 $n = 5$
64				0·066 ± 0·040 $n = 4$	0·044 ± 0·006 $n = 5$	0·042 ± 0·023 $n = 5$	0·029 ± 0·006 $n = 4$
88				0·124 ± 0·014 $n = 4$	0·089 ± 0·027 $n = 5$	0·088 ± 0·023 $n = 5$	0·077 ± 0·013 $n = 4$
95	0·095 ± 0·008 $n = 4$	0·103 ± 0·018 $n = 4$	0·128 ± 0·025 $n = 5$				
116	0·116 ± 0·024 $n = 5$	0·097 ± 0·010 $n = 5$	0·075 ± 0·017 $n = 5$				
143				0·207 ± 0·057 $n = 3$	0·200 ± 0·074 $n = 5$	0·200 ± 0·072 $n = 4$	0·161 ± 0·008 $n = 4$
165	0·165 ± 0·029 $n = 5$	0·170 ± 0·036 $n = 5$	0·136 ± 0·020 $n = 5$	0·259 ± 0·052 $n = 4$	0·145 ± 0·070 $n = 4$	0·200 ± 0·028 $n = 4$	0·203 ± 0·038 $n = 3$
176				0·173 ± 0·022 $n = 4$	0·184 ± 0·078 $n = 4$	0·094 ± 0·032 $n = 5$	0·166 ± 0·015 $n = 3$
196	0·163 ± 0·024 $n = 4$	0·144 ± 0·040 $n = 5$	0·178 ± 0·032 $n = 5$				
301				0·575 ± 0·117 $n = 3$	0·842 ± 0·360 $n = 4$	0·687 ± 0·155 $n = 4$	0·468 ± 0·184 $n = 3$
330	0·197 ± 0·073 $n = 4$	0·221 ± 0·033 $n = 4$	0·285 ± 0·064 $n = 4$				
349				0·468 ± 0·113 $n = 3$	0·715 ± 0·103 $n = 3$	0·177 ± 0·011 $n = 3$	0·595 ± 0·253 $n = 3$
378	0·566 ± 0·178 $n = 4$	0·177 ± 0·013 $n = 4$	0·207 ± 0·122 $n = 4$				
405					1·593 ± 0·717 $n = 3$	0·248 ± 0·049 $n = 4$	0·956 ± 0·503 $n = 3$
476				0·728 ± 0·249 $n = 3$			
506	0·372 ± 0·090 $n = 5$	0·157 ± 0·041 $n = 3$	0·058 ± 0·041 $n = 2$				
Pit. Wei.	13·0 ± 4·6 $n = 5$	12·0 ± 0·8 $n = 4$	13·0 ± 1·4 $n = 2$	20·0 ± 4·8 $n = 4$	24·5 ± 5·2 $n = 4$	15·6 ± 1·4 $n = 4$	15·0 ± 5·6 $n = 4$
Pit. Con.	1·548 ± 0·300 $n = 5$	0·405 ± 0·024 $n = 4$	0·322 ± 0·138 $n = 2$	9·128 ± 2·44 $n = 4$	14·58 ± 7·46 $n = 4$	5·88 ± 3·43 $n = 4$	37·20 ± 16·41 $n = 4$

Table 2

Day	orch. ♂♂ + P.I. + oestr.	orch. ♂♂ + P.I.	orch. ♂♂	orch. ♂♂ + oestr.	orch. ♂♂ + Clpr + oestr.	orch. ♂♂ + Clpr	♂♂ + Clpr
7	0·056 ± 0·023 $n = 4$	0·018 ± 0·011 $n = 4$	0·023 ± 0·009 $n = 5$	0·463 ± 0·171 $n = 4$	0·246 ± 0·067 $n = 4$	0·119 ± 0·062 $n = 5$	0·137 ± 0·017 $n = 4$
14	0·043 ± 0·011 $n = 4$	0·035 ± 0·017 $n = 4$	0·111 ± 0·109 $n = 4$	0·263 ± 0·130 $n = 4$	0·506 ± 0·086 $n = 5$	0·099 ± 0·052 $n = 5$	0·098 ± 0·040 $n = 4$
22	0·131 ± 0·018 $n = 4$	0·160 ± 0·049 $n = 4$	0·123 ± 0·040 $n = 4$				
25	0·146 ± 0·046 $n = 4$	0·113 ± 0·031 $n = 5$	0·083 ± 0·030 $n = 5$				
32	0·204 ± 0·179 $n = 3$	0·024 ± 0·020 $n = 3$	0·024 ± 0·015 $n = 5$				
50	0·149 ± 0·061 $n = 4$	0·010 ± 0·002 $n = 5$	0·010 ± 0·002 $n = 4$	0·015 ± 0·006 $n = 4$	0·088 ± 0·032 $n = 5$	0·021 ± 0·006 $n = 5$	0·010 ± 0·002 $n = 5$
64				0·310 ± 0·108 $n = 5$	0·490 ± 0·080 $n = 5$	0·128 ± 0·027 $n = 5$	0·108 ± 0·019 $n = 5$
88				0·269 ± 0·009 $n = 4$	0·480 ± 0·168 $n = 4$	0·118 ± 0·017 $n = 5$	0·092 ± 0·016 $n = 5$
95	0·116 ± 0·032 $n = 4$	0·065 ± 0·016 $n = 3$	0·084 ± 0·024 $n = 5$				
116	0·130 ± 0·023 $n = 5$	0·166 ± 0·049 $n = 4$	0·095 ± 0·022 $n = 5$				
143				0·276 ± 0·025 $n = 3$	0·373 ± 0·160 $n = 5$	0·123 ± 0·041 $n = 3$	0·272 ± 0·062 $n = 5$
165	0·218 ± 0·031 $n = 5$	0·131 ± 0·033 $n = 5$	0·123 ± 0·037 $n = 5$	0·428 ± 0·088 $n = 4$	0·366 ± 0·079 $n = 4$	0·259 ± 0·032 $n = 5$	0·223 ± 0·030 $n = 5$
176				0·268 ± 0·045 $n = 4$	0·392 ± 0·166 $n = 4$	0·212 ± 0·034 $n = 5$	0·171 ± 0·022 $n = 5$
196	0·383 ± 0·027 $n = 4$	0·191 ± 0·046 $n = 4$	0·215 ± 0·054 $n = 5$				
301				0·501 ± 0·068 $n = 3$	—*	0·291 ± 0·055 $n = 5$	0·638 ± 0·152 $n = 5$
330	0·317 ± 0·063 $n = 5$	0·090 ± 0·012 $n = 5$	0·090 ± 0·009 $n = 4$				
349				0·628 ± 0·135 $n = 3$	—	0·595 ± 0·205 $n = 4$	0·522 ± 0·079 $n = 4$
378	0·277 ± 0·061 $n = 5$	0·128 ± 0·041 $n = 4$	0·142 ± 0·007 $n = 4$				
405						0·283 ± 0·114 $n = 4$	0·317 ± 0·091 $n = 4$
476				0·666 ± 0·099 $n = 4$			
506	0·424 ± 0·272 $n = 4$	0·171 ± 0·073 $n = 3$	0·087 ± 0·007 $n = 3$				
Pit. Wei.	16·7 ± 2·3 $n = 4$	14·2 ± 2·9 $n = 5$	13·0 ± 1·8 $n = 4$	20·0 ± 11·6 $n = 4$	—	17·0 ± 1·6 $n = 5$	9·7 ± 1·7 $n = 5$
Pit. Con.	0·944 ± 0·430 $n = 4$	0·284 ± 0·069 $n = 5$	0·170 ± 0·038 $n = 4$	0·480 ± 0·201 $n = 3$	—	1·52 ± 0·15 $n = 5$	6·06 ± 3·73 $n = 3$

*This group of rats were killed accidentally, during a rearrangement of the animal house.

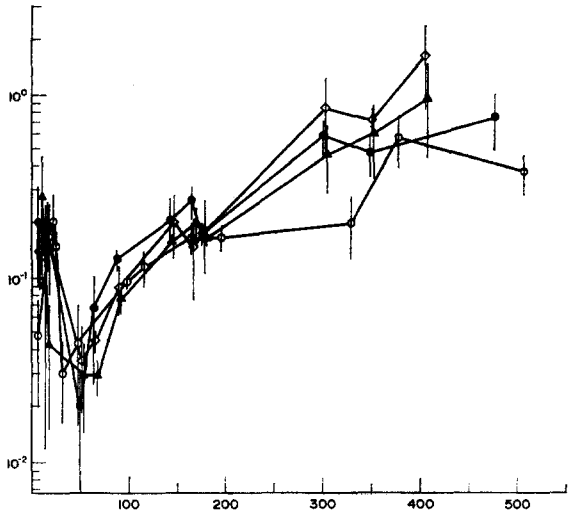

Fig. 4. Prolactin levels in plasma of 3 groups of spayed rats and 1 group of intact female rats. Further details see Fig. 3.

(*d*) ▲ *Intact female rats+375 mg Chloorpromazine/l. drinking water.*

(*e*) ○ *Spayed rats+pituitary under renal capsule+2 mg oestrone/l. drinking water.*

(*f*) ● *Spayed rats+2 mg oestrone/l. drinking water.*

(*g*) ◇ *Spayed rats+(375 mg chloorpromazine+2 mg oestrone)/l. drinking water.*

genous oestrogens or supplemented oestrone essentially show the same pattern over the first hundred days as the spayed rats represented in Fig. 3. However, in the four oestrogen groups represented in Fig. 4 the slow increase of the mean plasma prolactin values, which all seven groups of female rats exhibit in the period between day 100 and day 200 of the respective treatments, continues further in at least three of the four groups represented in Fig. 4 during the next 200–300 days, reaching mean values of well over 0·45 μg/ml.

On the day of autopsy (day 405 for groups d and g, day 476 for group f and day 506 for group e) the difference in weight of the *in situ* pituitaries was not statistically valid between the groups of the intact female rats treated with chloorpromazine (group d 15·0 ± 5·6 mg) the spayed female rats bearing a pituitary isograft and treated with oestrone (group e 13·0 ± 4·6 mg) and the spayed female rats treated with oestrone (group f 20·0 ± 4·8 mg). The pituitary weights of groups d and e were statistically significantly less than those of the spayed rats treated with both oestrone and chloorpromazine (group g 24·5 ± 5·2 mg; d<g, $0{\cdot}02<p<0{\cdot}05$; e<g, $p<0{\cdot}01$). In this regard group f did not differ from group g.

It was striking that the difference in weight of the pituitaries in the groups was not reflected by an equal difference in pituitary prolactin concentration (μg/mg w.w.). The pituitaries of group d had the highest prolactin concentration (37·20 ± 16·41), which was significantly different from group e (1·548 ± 0·300), group f (5·88 ± 3·43) and group g (14·58 ± 7·46; d>e, $p<0{\cdot}01$; d>f, $0{\cdot}01<p<0{\cdot}02$; d>g, $0{\cdot}02<p<0{\cdot}05$). The groups f and g were statistically not different, but both groups had a significantly higher pituitary prolactin concentration than group e (f>e, $p<0{\cdot}001$; g>e, $p<0{\cdot}01$).

Experiments with orchidectomized rats without oestrogen supplementation

Experiments h, i, j. As is shown in Fig. 5 the general pattern again resembles that of the spayed rats without oestrogen supplementation (Fig. 3), except for the finding that it apparently takes 2 to 3 weeks before the low plasma prolactin values of untreated male rats reach values of around 0·1 μg/ml after castration, in both groups of orchidectomized animals either with or without isografted pituitary tissue under their renal capsule.

The orchidectomized rats receiving chloorpromazine treatment differ from the two mentioned orchidectomized groups in two

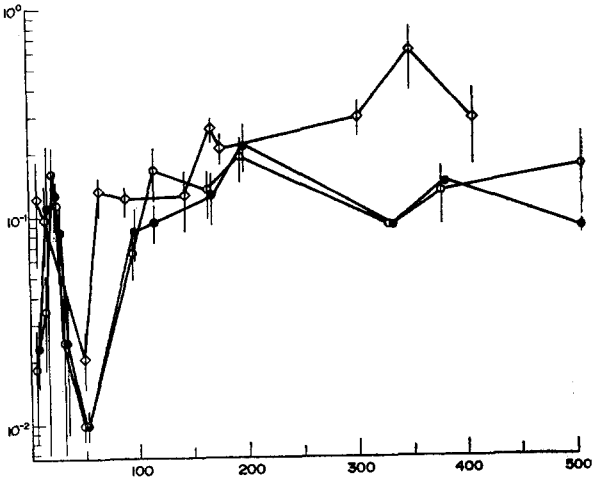

Fig. 5. Prolactin levels in plasma of orchidectomized rats under various experimental conditions. Further details see Fig. 3.

(*h*) ● *Orchidectomized rats.*

(*i*) ○ *Orchidectomized rats+pituitary under renal capsule.*

(*j*) ◇ *Orchidectomized rats+375 mg chloorpromazine/l. drinking water.*

aspects; (a) apparently as a result of chloorpromazine administration the mean plasma prolactin levels reach the mean value of around 0·1 μg/ml already on day 70 of the experiment, and (b) during the period of longer than 300 days of treatment the mean prolactin levels tend to increase further and are statistically significantly higher than those of the other two orchidectomized groups.

The difference in weight of the *in situ* pituitary glands between groups h, i and j on the day of autopsy (day 506 for groups h and i; day 405 for group j) was statistically not significant for the groups of the orchidectomized rats (group h: 13·0 ± 1·8 mg) and the orchidectomized rats treated with chloorpromazine (group j: 17·0 ± 1·6 mg) on the one hand and the orchidectomized rats bearing a pituitary isograft (group i: 14·2 ± 2·9 mg) on the other. However, the difference between groups h and j was statistically significant (h<j, $p<0·01$).

A significant difference in pituitary weight has not been noticed between male groups h, i and j and the appropriate female groups a, b and c.

The prolactin concentration (μg/mg w.w.) of the pituitary glands differed significantly between group h (0·170 ± 0·038) and group i (0·284 ± 0·069; h<i, $0·02<p<0·05$). Both groups were significantly different from group j (1·52 ± 0·15; h<j, i<j, $p<0·001$).

The difference in pituitary prolactin concentration between this three groups of male rats and the appropriate female groups a, b and c was statistically significant between groups i and j on the one hand and groups b and c on the other (i<b, j<c, $0·01<p<0·02$).

Experiments with intact male rats treated with chloorpromazine and with orchidectomized rats with oestrone supplementation

Experiments k, l, m, n. A more or less pronounced dip in the plasma prolactin values at or around day 50 of the experiment was observed in all groups with the exception of the orchidectomized rats bearing a pituitary isograft and treated with oestrone. In this last group the mean plasma prolactin values showed a more or less steady increase during the first 32 days; as can be seen, however, over the whole period between day 22 and day 165 of the experiment the plasma values were more or less stabilized, fluctuating between 0·1 and 0·2 μg/ml. Apparently then the mean plasma levels increased further to become stabilized again at a higher mean value, since they fluctuated around mean values of 0·35 μg/ml over the period of observation covering the last 300 days of the experiment.

The intact male rats treated with chloorpromazine had mean plasma values of around 0·1 μg/ml at days 7 and 14, returned to this range of values after the dip, fluctuated around 0·2 μg/ml over the second 100 days period to increase still further to reach a mean plasma value of over 0·6 μg/ml at day 301 of the experiment, which was followed by a decrease till the day of autopsy (day 405).

The orchidectomized rats supplemented with oestrone reached fairly high mean plasma levels already at day 7, showed a pronounced dip at day 50, returned to fairly high levels after this dip fluctuating for more than 100 days between mean plasma values of 0·27 and 0·43 μg/ml and showed a further increase when the plasma values were more than 0·5 μg/ml during the last 175 days of the experiment.

The orchidectomized rats treated with the combination of oestrone and chloorpromazine showed a mean plasma value of 0·5 μg/ml at day 14, dropped to 0·09 μg/ml on day 50 and fluctuated thereafter between mean values of 0·5 μg/ml and 0·36 μg/ml for the next 100 days. Unfortunately the rats of this experimental group died accidentally a few weeks after the assay series at day 176 of the experiment.

On the day of autopsy (day 405 for group k; day 506 for group l; day 476 for group m) the difference in weight of the *in situ* pituitary glands was not statistically significant between the orchidectomized rats treated with oestrone (group m 20·0 ± 11·6 mg) on the one hand and both the orchidectomized rats bearing a pituitary isograft and treated with oestrone (group l 16·7 ± 2·3 mg) and the intact male rats treated with chloorpromazine (group k: 9·7 ± 1·7 mg) on the other. The large standard deviation in

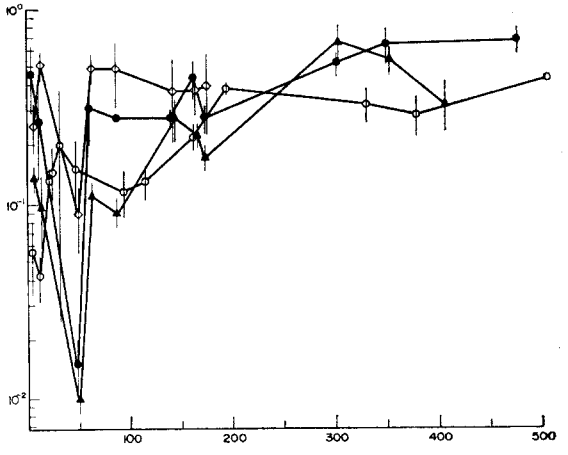

Fig. 6. Prolactin levels in plasma of 3 groups of orchidectomized rats and 1 group of intact male rats. Further details see Fig. 3.

(*k*) ▲ *Intact male rats + 375 mg chloorpromazine/l. drinking water.*

(*l*) ○ *Orchidectomized rats + pituitary under renal capsule + 2 mg oestrone/l. drinking water.*

(*m*) ● *Orchidectomized rats + 2 mg oestrone/l. drinking water.*

(*n*) ◇ *Orchidectomized rats + (375 mg chloorpromazine + 2 mg oestrone)/l. drinking water. This group died accidentally on day* 215.

group m was caused by a tumorous outgrowth of the *in situ* pituitary gland in one of the animals, weighing 37 mg. Group 1 was significantly different from group k ($1>k$, $p<0{\cdot}01$).

A significant difference in pituitary weight has not been noticed between the male groups k, l and m and the appropriate female groups d, e and f.

The discrepancy between pituitary prolactin concentration and weight already mentioned for the female group d was even more pronounced in the male group k. The smallest pituitaries showed the highest prolactin concentration ($6{\cdot}06 \pm 3{\cdot}73$ μg/mg w.w.). This was significantly higher than all the other male groups. When only the oestrone receiving males are compared: group 1 ($0{\cdot}944 \pm 0{\cdot}430$ μg/mg w.w; $k>1$, $0{\cdot}01<p<0{\cdot}02$), group m ($0{\cdot}480 \pm 0{\cdot}201$ μg/mg w.w; $k>m$, $0{\cdot}02<p<0{\cdot}05$).

The difference in pituitary prolactin concentration between this three groups of male rats and the appropriate female groups d, e and f was statistically significant ($k<d$, $l<e$, $0{\cdot}02<p<0{\cdot}05$; $m<f$, $p<0{\cdot}01$).

The effects of the administration of bovine prolactin on endogenous prolactin levels studies in three experimental groups.

The disappearance rate of bovine prolactin injected into a tail vein from the general circulation was studied by radioimmunoassay of samples of plasma taken at 20, 40 and 80 min after the injection. The effects of the administered heterologous prolactin on the endogenous prolactin levels were followed by radioimmunoassay of plasma samples taken at the time of injection and of the same samples mentioned for the assay for the bovine hormone. The results of the various radioimmunoassays are summarized in Table 3. The value for bovine prolactin at the time of injection are given as 20 μg/ml assuming immediate equilibration of the administered dose and a blood volume of 15 ml per rat.

The mean bovine prolactin values assayed in the 20-min samples did not differ statistically among the three groups of rats studied. However, in the samples taken at 40 and at 80 min the rats receiving oestrone in their drinking water appeared to eliminate the administered bovine hormone at a statistically significantly faster rate than the rats of the two other groups (all four possible comparisons showed $p<0{\cdot}01$ with Student's *t*-test), whereas the mean plasma values at 40 and 80 min between the two groups not receiving oestrone did not differ in a statistically valid way ($0{\cdot}20<p<0{\cdot}30$). Moreover the slopes of the assays of the rats receiving oestrone at 40 and at 80 min were flatter than the slopes of the reference curves, whereas all slopes of the assays for the rats not receiving oestrone were all parallel to the reference curves. It suggests that the bulk of the administered bovine prolactin in the oestrone-treated rats had been broken down into immunoreactive fragments within 40 min.

The effect of the administered heterologous hormone on the endogenous prolactin levels can be summarized as an accentuation of the inter-group differences; whereas the endogenous prolactin levels differed significantly only between the group receiving oestrone and the two other groups (both comparisons: $0{\cdot}01<p<0{\cdot}02$) at the time of injecting the bovine hormone, the differences between the castrated rats bearing an isograft and the castrated rats were statistically not valid ($0{\cdot}40<p<0{\cdot}50$).

Table 3. Disappearance rate of bovine prolactin and the effect of administered heterologous hormone on the endogenous prolactin levels

Endocrine state	Time in minutes after administration of 300 μg bovine prolactin			
	0 μg/ml	20 μg/ml	40 μg/ml	80 μg/ml
Bovine prolactin: castrated ♂ + isograft + oestrone	20*	$1{\cdot}97 \pm 0{\cdot}58$	'$0{\cdot}089 \pm 0{\cdot}067$'	'$0{\cdot}064 \pm 0{\cdot}026$'
Endogenous hormone:	$0{\cdot}365 \pm 0{\cdot}082$	$0{\cdot}584 \pm 0{\cdot}057$	$0{\cdot}568 \pm 0{\cdot}051$	$0{\cdot}624 \pm 0{\cdot}047$
Bovine prolactin: castrated ♂ + isograft	20*	$1{\cdot}90 \pm 0{\cdot}38$	$1{\cdot}195 \pm 0{\cdot}135$	$0{\cdot}495 \pm 0{\cdot}060$
Endogenous hormone:	$0{\cdot}239 \pm 0{\cdot}022$	$0{\cdot}330 \pm 0{\cdot}034$	$0{\cdot}364 \pm 0{\cdot}021$	$0{\cdot}358 \pm 0{\cdot}051$
Bovine prolactin: castrated ♂	20*	$1{\cdot}82 \pm 0{\cdot}72$	$1{\cdot}06 \pm 0{\cdot}19$	$1{\cdot}045 \pm 0{\cdot}065$
Endogenous hormone:	$0{\cdot}211 \pm 0{\cdot}062$	$0{\cdot}261 \pm 0{\cdot}018$	$0{\cdot}324 \pm 0{\cdot}016$	$0{\cdot}246 \pm 0{\cdot}023$

*Assuming immediate equilibration of administered dose.
'··· ± ···' slope not parallel to reference curve, but "flatter"

However, the differences between the three groups of rats at 20, 40 and 80 min were all statistically significant (the difference between the two groups not receiving oestrone at 40 min was significant at $0{\cdot}01<p<0{\cdot}02$; all other comparisons differed significantly at $p<0{\cdot}01$).

Followed in time, the effects of heterologous hormone injection on endogenous prolactin levels showed, for both groups of rats bearing isografts, an immediate rise in the fi st 20 min to levels, which differed statistically significantly ($p<0{\cdot}01$) from those at time 0. The mean plasma levels in both these groups fluctuated but little for the next hour (for both groups differences between levels assayed at 20 min and those found at 40 and 80 min respectively were statistically not valid). The rise in mean prolactin levels in the castrated rats without an isograft was slower (difference between values at 0 and 20 min: $0{\cdot}05<p<0{\cdot}10$; between 0 and 40 min: $p<0{\cdot}01$) and of shorter duration, since the levels of prolactin in the circulation at 80 min were already lower again (difference between values at 40 and 80 min: $p<0{\cdot}01$). In fact they had returned to the initial values, since the values at 80 min did not differ statistically ($0{\cdot}30<p<0{\cdot}40$) from the values assayed at 0 min.

Effects of fortnightly oestrone pellet implantation in orchidectomized rats bearing a pituitary isograft

Experiment o. The observations, e.g. plasma prolactin and *in situ* pituitary prolactin assay results, weights of the pituitary glands *in situ* as well as of the isografts, localization of mammary tumours, made in this group of rats are summarized in Table 4. The plasma prolactin values of day 201 of the experiment are close together, having a mean value of 2·8 μg/ml and a range of 2·4 to 3·2 μg/ml. In view of the pituitary weights on day 321 of the glands *in situ* and the remarkable constancy of pituitary growth rate in mice and rats when the pituitary cells have begun to proliferate [17], we have reasons to believe that the glands *in situ* were not yet in the geometrical proliferation stage on day 201. Since the plasma values on day 201 were significantly higher than those observed earlier [13] in comparable rats not bearing pituitary isografts and before the pituitary cells entered the proliferation stage during oestrone stimulation, the higher levels in the circulation observed in the present experiment suggested an appreciable contribution by the isografts.

The finding that after 321 days of the oestrone regimen the pituitary glands *in situ* attained much larger sizes than the isografts indicated that the isografted pituitary tissue went into the proliferation stage at a much later time than the glands *in situ*. The fact that by far the highest plasma level was observed in the rat having the largest (375 mg) *in situ* pituitary tumour indicated that at this later stage of the experiment the size of the pituitary tumour *in situ* determined the plasma levels of prolactin rather than the relatively smaller contribution of the much smaller isografts.

It should be noted that three (i.e. the rat that died on day 235 and two of the rats that were killed at the termination of the experiment) out of five rats developed mammary tumours before the age of 400 days.

DISCUSSION

The induction of prolactin producing pituitary tumours in the *in situ* glands by continuous administration of adequate doses of oestrogen is successful in many strains of mice and rats (for review see Russfield [1]). In our laboratory

Table 4. Observations in rats bearing isografts and implanted fortnightly one oestrone pellet. Experiment o

	Rat A	Rat B	Rat C	Rat D	Rat E
Prolactin level on day 201	2·4 μg/ml	2·5 μg/ml	2·9 μg/ml	3·0 μg/ml	3·2 μg/ml
Prolactin level on day 321	116·9 μg/ml	—*	29·1 μg/ml	48·1 μg/ml	33·1 μg/ml
In situ					
Pituitary prolactin concentration	4·090 μg/mg	—	4·835 μg/mg	4·830 μg/mg	8·823 μg/mg
Pituitary *in situ*	375 mg	—	93 mg	112 mg	91 mg
Pituitary isograft	25 mg	—	25 mg	15 mg	45 mg
Mammary tumour	—	L_2	R_6	R_2	—

*Rat B died on day 235 of the experiment.

the method of choice in *mice* is the administration of oestrone dissolved in drinking water. For this purpose oestrone has to be given at its maximum solubility in water (2 mg/l.). Reduction of the dose to 1 mg of oestrone/l. of drinking water prolongs the induction time with 100 to 200 days [17]. Once the pituitary glands start to increase in weight, however, the time necessary for the glands to double their weight is exactly the same as that observed in the same strain of mice with the higher dose of oestrone. A further decrease of oestrone (e.g. $\frac{1}{2}$ mg/l.) fails altogether to induce cellular proliferation in the *in situ* pituitary gland. In *rats* oestrone dissolved in drinking water appears not to be effective and one has to resort to other ways of administering this hormone for the induction of pituitary tumours. This is not surprising when it is realized that maximum solubility of oestrone corresponds to a 16-fold higher dose than to restore in spayed mice vaginal cornification, whereas for rats it is only 4-fold higher than the dose that restores vaginal cornification in the spayed animal. Whether this difference reflects a more efficient break-down by the liver of the rat rather than greater sensitivity of the relevant target tissues for oestrone in mice awaits oestrogen level estimates in mice and rats under various experimental conditions.

When the prolactin estimates in the circulation of rats treated with adequate doses of oestrone for the induction of primary pituitary tumours [13] are compared with the assay results in mice developing oestrone-induced primary pituitary tumours, two differences—apart from the difference in the dose of oestrone and in the way of administration—seem to be of importance: (1) the much longer period during which the pituitary gland in the *rat* needed to be stimulated to excessive prolactin secretion before the prolactin producing cells reacted with cellular proliferation accompanied by a further increase in plasma prolactin; and (2) the difference between the rates of progressive increase in pituitary weights and in plasma prolactin levels: in *mice* over quite a long period, the time needed for the pituitary weights to become twice as high was exactly the same as the time needed for the plasma prolactin content to do so, whereas in *rats* a 15- to 20-fold increase in pituitary weight was accompanied by a 5- to 8-fold increase in plasma prolactin levels.

The fact that it was possible to confirm the earlier plasma prolactin estimates in isograft-bearing mice [18] by repeating the estimates in isograft-bearing mice [18] by repeating the estimates with a radioimmunoassay, which employed a well defined rat prolactin preparation as the ^{125}I–labelled hormone, (the antiserum raised against mouse prolactin employed in the earlier experiments and the mouse hormone preparation for reference curves were used) suggests that the hormone estimated by both systems is indeed prolactin. It is also an indication that any laboratory having access to rat-prolactin preparations can carry out mouse plasma estimates. Pilot experiments have indicated that it should be possible to employ the serum of a rabbit that has been injected with crude pituitary homogenates of mice treated with chloorpromazine to increase the pituitary prolactin content.

When the exponential increase in pituitary tissue of the isografts in female $(C_{57}BL \times CBA)F_1$ hybrid mice is compared to that of the *in situ* pituitary glands in oestrone treated orchidectomized $(C_{57}BL \times DBA_f)F_1$ hybrid mice, the weight curves match exactly. Gardner [20, 21] suggests that the rate of cellular proliferation is determined by the relevant dominant gene of the $C_{57}BL$.

The assay results in orchidectomized mice bearing isografts indicate that in mice a dose of oestrone, four times the dose necessary to restore vaginal cornification in spayed mice is sufficient to induce the isograft (i.e. pituitary tissue uninhibited by PIF) to pour out prolactin into the general circulation at such a rate that the prolactin producing cells are induced to proliferate. Evidence was obtained that even much smaller doses of oestrone in drinking water (0·0125 mg oestrone/l.) than necessary to restore vaginal cornification resulted in a progressive increase in isografted pituitary tissue as well as in plasma prolactin values [22].

In sharp contrast to the relative ease to induce ectopically implanted pituitary tissue to such a hyperactivity of the prolactin producing cells that proliferation of the cells ensues in *mice*, isografted pituitary tissue in *rats* react to oestrone administration in their drinking water (dose of oestrone four times the amount needed to restore vaginal cornification in spayed rats) with an increase of prolactin secretion resulting in plasma prolactin levels, which are definitely lower than the levels occurring in pro-oestrus or oestrus in normal cycles of female rats. Apparently the degree of activity of the prolactin producing cells in the isograft under these conditions (i.e. released from the inhibitive action of PIF concurrent with super-normal oestrogen levels) is not sufficiently increased as to trigger off cellular proliferation.

The more or less pronounced dip of the plasma prolactin level noticed in practically all groups of rats on the 50th day of the experiment, appears not be an isolated phenomenon. In another set of experiments [23] the first 100 days are studied more closely. During the first 10 weeks a rise and fall of the plasma prolactin levels frequently occur, probably due to the adaptation of the animals to the experimental conditions.

When plasma levels in any of the groups of rats bearing isografts receiving oestrone are compared with those in corresponding groups of non-isograft bearing animals no consistently differing mean plasma levels were observed; at several occasions the plasma levels of oestrone treated non-isograft bearing rats were even significantly higher than the comparable isograft-bearing animals.

In electron microscopic studies, kindly performed by Dr. Feltkamp, the activity of the prolactin cells and the secretion rate of prolactin is higher in the *in situ* pituitary than in the isograft as judged by the presence of the development of the endoplasmatic reticulum and the number of hormone granules. This is not consistent with the findings of Mena *et al.* [24], in which the isograft is thought to secrete such large amounts of prolactin, that the prolactin secretion by the *in situ* pituitary is inhibited. In mice, however, Feltkamp [25] reported evidence of an inhibition of the prolactin cells in the *in situ* gland, presumably by the prolactin secreted by the isograft.

The working mechanism of chloorpromazine is comparable with hypophyseal stalk sectioning [26], since both result in a release of the prolactin cells from the inhibitory activity of the hypothalamus (PIF, prolactin inhibiting factor). One would expect that the resulting prolactin levels of the *in situ* pituitary gland in chloorpromazine treated rats and of the isografted gland in otherwise comparable groups of rats will not differ much. This seems more or less to be the case, however, in oestrone treated orchidectomized males the mean prolactin levels in the chloorpromazine treated group was definitely higher than in the isograft-bearing group around day 100 of treatment, and in the oestrone treated spayed rats the prolactin level in the chloorpromazine treated group tended to be consistently higher than the levels in the isograft bearing rats from day 200 of the experiment onward. Moreover, the isografts on the day of autopsy were not larger than the isografts in other groups of rats (the isografts give the impression to have become smaller in size than at the time of grafting; being so small in size and so intimately related to the underlying kidney tissue, it was impossible to dissect them), the chloorpromazine–oestrone treated spayed rats had significantly enlarged *in situ* pituitaries (24·5 mg ± 5·2) with a relatively high prolactin concentration (14·6 μg/ml w.w. ± 7·5).

This suggests that in the absence of PIF the *in situ* pituitary reacts more vigorously to oestrone stimulation than the isograft. A further indication that this is not due to a specific and direct action of chloorpromazine on the prolactin producing cells comes from the results of oestrone pellet implantation; all the *in situ* pituitaries were enlarged and weighed more than 90 mg, whereas one isograft was slightly enlarged, two were enlarged to two or three times the size at the time of grafting and one was enlarged to 45 mg. Thus it may be postulated that *in the rat* the combination of oestrogen stimulation and absence of PIF does not stimulate the prolactin producing cells to such a degree that it triggers off cellular proliferation; only when on top of this a positive stimulus from the hypthalamus (PRF, prolactin releasing factor, or PSA, prolactin stimulating activity [27, 28]) acts on the pituitary cells of the rat the degree of stimulation triggers off proliferation of the prolactin producing cells. Presumably either the postulated hypothalamic factor in the high oestrone experiment was so much increased that even diluted in the general circulation it could act on the isograft, or the high dose of oestrone in combination with the absence of PIF was sufficient stimulation to induce cellular proliferation albeit much later than the combined action of the absence of PIF, the high oestrogen level and the positively acting hypothalamic factor on the cells in the *in situ* gland.

The relative value of plasma prolactin determinations as a parameter of prolactin secretory activity is also suggested by the significant differences found in rats of the various groups as to the mean weights of the *in situ* glands and their prolactin concentration.

In the rats which were not treated with oestrone, both male and female groups treated with chloorpromazine, developed the biggest *in situ* pituitaries which had the highest prolactin concentration. The increase of the plasma prolactin level, however, was only significant in the male group treated with chloorpromazine.

The plasma prolactin levels in the oestrone treated or in the intact rats, again showed a great resemblance in all groups. Perhaps a sex difference can be noticed, due to a pronounced effect of oestrone in male rats (e.g. the prolactin

concentration increased sooner to higher levels in oestrone treated male rats, than in female rats). The main differences, however, among the different oestrone treated and intact groups was found in the weight and prolactin concentration of the *in situ* pituitaries.

The male groups, though developing pituitaries of the same weight as the appropriate female groups, showed a significant lesser pituitary prolactin concentration than the female groups.

The intact animals of both series treated with chloorpromazine had the highest pituitary prolactin concentrations which were significantly higher than all the other groups. It was remarkable that the pituitaries of these animals were almost the smallest. All other groups showed a fair correlation between increase in pituitary weight and increase in pituitary prolactin concentration.

The relative importance of the plasma prolactine values is stressed again in the experiment describing the disappearance rate of bovine prolactin and the effect of the administration of this heterologous hormone on the endogenous prolactin levels.

Though the plasma prolactin levels at the beginning of this experiment were almost the same in castrated males, and castrated males bearing a pituitary isograft and treated with oestrone, the endogenous levels 20 min after administration of bovine prolactin showed a marked increase in the castrated rats bearing a pituitary isograft and treated with oestrone, a less outspoken increase in the castrated rats bearing an isograft only, and barely any increase in the castrated rats. The disappearance rate of bovine prolactin in the various groups was also different, being persistent in the castrated rats bearing an isograft and treated with oestrone, persistent also but at a lesser rate in isograft-bearing animals, but breaking off after 20 min in the castrated rats. It shows that the first mentioned group is accustomed in breaking down large quantities of prolactin in a short time, the second group also but to a lesser extent, and the third group is not accustomed to do so, indicating the degree by which the prolactin secretion rates may differ in the various groups. This experiment gives an idea about the real amounts of prolactin secreted by the pituitary gland.

Several experimental conditions are effective in increasing mammary tumour incidence *in mice*, e.g. force breeding, chronic oestrogen administration and pituitary isografts. The common denominator in all these conditions is hyperprolactinaemia over long periods of time. Before radioimmunoassay of prolactin made it feasible to verify actual prolactin levels, it was taken for granted that at least during the luteal phase of pregnancy prolactin levels are high and the change of the normal five-day oestrous cycle into a series of subsequent pseudopregnancy cycles by isografting a pituitary gland away from the hypothalamus was considered to be conclusive evidence for continuously increased prolactin levels in the circulation. Since chronic oestrogen treatment also resulted in pituitary tumours in various strains of mice and such tumours are always of the prolactin-producing type; the mode of action of increasing mammary tumour incidence in mice may well be explained to be an indirect one, i.e. by inducing the pituitary gland to secrete prolactin at super normal amounts.

Since neither force breeding nor implanting an isologous pituitary gland away from the hypothalamus appears to influence mammary tumour incidence appreciably, by the same inductive reasoning one may draw the conclusion that *in rats* prolactin is only marginally effective if at all in the induction of mammary tumours. The fact that chronic oestrogen treatment appears to be effective in both species may be explained by postulating a different mode of action, i.e. an indirect one in mice and a direct effect on the mammary gland in rats.

Evidence obtained by radioimmunoassay, however, suggest that in both species prolonged hyperprolactinaemia results in mammary tumours. The homeostatic mechanisms in the rat however, prevent in most experimental conditions a prolonged increase of prolactin levels in the circulation.

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Parallelism between Oestrogen Binding Capacity and Hormone Responsiveness of Mammary Tumours in GR/A Mice

L. TERENIUS

Department of Pharmacology, University of Uppsala, Uppsala, Sweden

Abstract—*Mammary tumours arise in the GR/A-mouse strain at pregnancy. Such tumours are initially pregnancy-dependent, i.e. regress after parturition. The tumours undergo progression: at subsequent pregnancies postpartum regression becomes less complete and finally growth is independent of hormones.*

The content of oestrogen 'receptors' in pregnancy-dependent and independent tumours of GR/A-mice has been determined. It was found that the dependent and non-regressing tumours contained such receptors while the independent tumours had low levels or absence of receptors.

The parallel loss of oestrogen receptors and hormone dependence suggest that oestrogens play a role for growth and control at the dependent stage.

SOME MAMMARY tumours in experimental animals depend on hormonal stimulus. This is the case of the pregnancy-dependent tumours in mice [1]. Breeding females of the inbred mouse-strain GR/A [2] usually develop such mammary tumours during their 2nd, 3rd or 4th pregnancies. The tumours grow rapidly during pregnancy but regress within one week after parturition. With following pregnancies the tumours reappear and may show less complete postpartum regression; eventually they continue to grow in non-pregnant animals. This progression from pregnancy-dependence to independence occurs independently in different tumours in the same animal [3, 4].

Following an initial observation that the pregnancy-dependent tumours in GR/A mice bound oestradiol–17β (i.e. contained oestrogen-'receptors'), the question was studied whether the development of autonomy was accompanied by loss of such receptors. This forms the content of the present paper.

MATERIAL AND METHODS

Inbred female mice of the strain GR/A [2] were used. They were subjected to forced breeding. Palpable pregnancy-dependent tumours usually appeared at the 2nd, 3rd or 4th pregnancies. The animals were inspected once weekly and the size and location of the tumours was noted.

The breeding animals were inspected for tumours also when they were no longer fertile. This was done at least every two weeks.

Material from pregnancy-dependent tumours was obtained at the time of parturition. Tumours in non-pregnant animals were usually obtained at autopsy. Such tumours had been observed for at least 1 month after the last parturition of the animal.

Determination of oestradiol–17β binding capacity was performed as described elsewhere [5]. Slices of the tumours (less than 0·5 mm thick and weighing 5–10 mg) were incubated with tritium-labelled oestradiol–17β in a Krebs–Ringer phosphate buffer, at pH 7·4 with 2% bovine albumin and hormones as described below. From each tumour, slices were distributed at random to a 'treatment' flask with 2×10^{-9}M labelled oestradiol–17β and to a 'carrier' flask which contained the same concentration of labelling material plus 2×10^{-7}M non-labelled oestradiol–17β. The flasks were incubated for 1 hr at 37°C in these solutions and then washed in the buffer cited

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above with 2×10^{-7}M non-labelled oestradiol–17β for 1 hr at 25°C to remove most of the non-specifically bound label.

Each tissue slice was digested with hyamine and the radioactivity determined by liquid scintillation. Counting efficiency was determined by external standardization.

The calculation of receptor-bound oestradiol–17β is based on the finite binding capacity of the specific binders and the knowledge that the used excess of non-labelled oestradiol–17β in 'carrier' flasks is saturating. Then, specifically bound oestradiol–17β relative to the medium concentration is

$$\frac{\text{dpm per mg 'treatment' tissue}}{\text{dpm per } \mu\text{l 'medium'}} - \frac{\text{dpm per mg 'carrier' tissue}}{\text{dpm per } \mu\text{l 'medium'}}.$$

where 'treatment' and 'carrier' refer to the different incubation flasks (see above) and 'medium' is the solution to which the label was added. This difference is used in the tables Significance was tested with the Student t-test.

RESULTS

Tumour slices were incubated with labelled oestradiol–17β and the oestradiol–17β binding capacity was determined (cf. Methods).

Material from tumours which appeared at pregnancy all bound oestradiol–17β significantly (Table 1). Biopsies of tumours were used in several cases to ascertain that the tumours regressed after parturition, i.e. were pregnancy-dependent. It is also apparent that the oestradiol–17β binding capacities were similar in all tumours, except in one which had a fairly low binding capacity.

Table 1. Oestradiol–17β binding capacity in pregnancy-dependent and pregnancy-stimulated mammary tumours

Mouse No.	Tumour	Oestradiol–17β binding capacity
32	Pregnancy-dependent*	$1{\cdot}03 \pm 0{\cdot}16$‡[1]
6	Pregnancy-dependent*	$0{\cdot}89 \pm 0{\cdot}11$[1]
9	Pregnancy-dependent*	$0{\cdot}79 \pm 0{\cdot}06$[1]
12	Pregnancy-dependent*	$0{\cdot}69 \pm 0{\cdot}08$[1]
13	Pregnancy-dependent*	$0{\cdot}67 \pm 0{\cdot}04$[1]
71	Pregnancy-dependent*	$0{\cdot}42 \pm 0{\cdot}16$[3]
22	Pregnancy-stimulated†	$1{\cdot}09 \pm 0{\cdot}26$[2]
23	Pregnancy-stimulated†	$0{\cdot}76 \pm 0{\cdot}10$[1]
2	Pregnancy-stimulated†	$0{\cdot}70 \pm 0{\cdot}06$[1]
21	Pregnancy-stimulated†	$0{\cdot}69 \pm 0{\cdot}14$[2]

*Outgrowth during pregnancy, complete regression after parturition (biopsy studied).
†Outgrowth during pregnancy (whole tumour excised).
‡Mean values ± s.e.m. are given. Ten to twelve slices per tumour were studied.
[1] = $p < 0{\cdot}001$; [2] = $p < 0{\cdot}01$; [3] = $p < 0{\cdot}05$.

Secondly, pregnancy-independent tumours were studied. Their growth patterns could be characterized as follows: (1) tumours which grew out during pregnancy but (a) did not regress and remained fairly static or (b) continued to grow; (2) tumours which did not grow out during a pregnancy and were growing at the time of study. The growth rate of growing tumours varied widely and was sometimes difficult to estimate since several independent tumours became necrotic or highly vascular. However, the present material only includes tumours which mainly contained living tissue. Differences in growth rates were therefore real. It was found that tumours which appeared during pregnancy and did not regress at parturition bound oestradiol–17β significantly (Table 2, upper part) and that their binding capacities were equal to those of the pregnancy-dependent tumours (cf. Table 1). Steadily growing tumours, either not regressing after pregnancies or growing out when the animal was not pregnant, had less oestradiol–17β binding capacity (Table 2, lower part). Several of these tumours were rapidly growing, had a solid structure and contained almost exclusively apparently living tissue. A number of these tumours continued to grow also in castrated animals.

The morphology of several tumours was studied. Pregnancy-dependent tumours and the static tumours with estrogen-binding properties (20L1, 1L5, 4L4, 9R3 in Table 2) were morphologically of the type P [6] while all sectioned independent tumours were adenocarcinomas. The same relation between tumour morphology and hormone-dependency has been found by van Nie and Dux [6].

DISCUSSION

Most mammary tumours in mice are, once palpable, no longer dependent on hormonal stimulus [7]. However, in some strains of mice, female breeders develop mammary tumours which regress after parturition [1–3, 8, 9]. Such tumours are called pregnancy-dependent. During subsequent pregnancies the tumours reappear but become increasingly more autonomous and finally grow in non-pregnant animals.

Van Nie and Thung [4] observed that tumours in the GR/A mouse which regressed after pregnancy could be reactivated by combined treatment with oestrone and progesterone, but that neither of these two hormones alone was able to reactivate growth. The growth of the tumours during pregnancy

Table 2. Oestradiol-17β binding capacity in non-growing and growing pregnancy-independent mammary tumours

Mouse No./ Tumour		Characteristics of the tumour: Start	Growth rate	Remarks	Oestradiol-17β binding capacity
31	L4*	P†	−‡	No regress after pregnancy	1·61 ± 0·29§[1]
20	L1	P	−	No regress after pregnancy	1·09 ± 0·15[1]
1	L5	P	−	Present during 2 pregnancies, some increase in each, no regress in-between	0·93 ± 0·14[1]
4	L4	P	−	No regress after pregnancy	0·90 ± 0·18[2]
9	R3	P	+	Present during 2 pregnancies. No regress after the 1st pregnancy, some increase during the next one	0·48 ± 0·19[3]
1	R3	P	+ +	No regress after pregnancy	0·30 ± 0·12[3]
8	L3	O	+ + +	Earlier pregnancy-dependent tumour at the same site	0·24 ± 0·14
9	R2	O	+ + +	Earlier pregnancy-dependent tumour at the same site. Castrated 10 days before tumour excision	0·21 ± 0·06[2]
3	L2	O	+	Castrated 7 days before excision	0·13 ± 0·04[3]
20	L5	O	+ + +	Earlier pregnancy-dependent tumour at the same site	0·13 ± 0·13
34	L4	O	+ +		0·12 ± 0·09
13	L1	O	+ +	Castrated 10 days before excision	0·06 ± 0·14
23	L5	P	+	No regress after pregnancy. Castrated 12 days before excision	0·04 ± 0·03
71	L2	P	+ +	Castrated 10 days before excision	−0·05 ± 0·09
24	L5	O	+	Castrated 10 days before excision	−0·11 ± 0·20

*L4 = is 4th mammary gland on left side R3 3rd mammary gland on right side etc.

†P = outgrowth during pregnancy; last pregnancy at least 1 month before tumour excision. O = outgrowth not associated with pregnancy.

‡− = static; + = increase in tumour volume < 5 times, + + = 5–50 times and + + + = > 50 times during last month before tumour excision.

§Mean values ± s.e.m. are given. There were at least 10–12 slices per tumour. [1] = $p < 0{\cdot}001$; [2] = $p < 0{\cdot}01$ [3] = $p < 0{\cdot}05$.

is therefore directly or indirectly attributable to elevated levels of these hormones. The presence of oestrogen-binding capacity in the pregnancy-dependent tumours indicates that oestrogens directly influence these tumours.

Also in the DMBA*-induced mammary cancer in female rats oestrogen binding capability runs parallel with hormone dependency. Thus, DMBA-induced tumours which do not regress after ovariectomy concentrate oestradiol–17β less efficiently than the regressing tumours [10, 11]. Like the pregnancy-dependent mouse tumours the DMBA tumours in rats accelerate at pregnancy and tend to regress during lactation [12, 13].

Oestrogen binding occurs only to the target tissues of these hormones [14]. The presence of specific oestrogen binders in hormone-dependent DMBA tumours of rats, pregnancy-dependent tumours in GR/A mice as well as in oestrogen-dependent hamster kidney tumours [15] makes it highly probable that these tissues are also target tissues for oestrogens and directly stimulated by oestrogens. Further support for this hypothesis comes from the parallel loss of hormone dependency and loss of oestrogen binders. However, other hormones certainly also provide stimuli for mammary tumour growth. The precise role of oestrogens in this concerted hormonal action has yet to be elucidated.

*DMBA = 7,12-dimethylbenz(a)anthracene.

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Prolonged Survival of Skin Allografts from Tumour-bearing Mice*

L. MILAS, M. HORVAT and V. SILOBRČIĆ†

Department of Biology, "Rudjer Bošković" Institute, and Central Institute for Tumours and Allied Diseases, Zagreb, Croatia, Yugoslavia

Abstract—*Skin grafts from C57BL mice bearing methylcholanthrene-induced fibrosarcoma or Ehrlich ascites carcinoma (EAC) survived longer in normal A mice than the grafts of skin from healthy C57BL mice. Fibrosarcoma showed this effect only after it had enlarged to a diameter of about 9 mm, whereas EAC was effective 3 days after inoculation. Second-set grafts of skin from EAC bearers survived longer even in mice presensitized with normal skin. On the other hand, second-set grafts of normal skin were rejected as quickly in mice grafted first with EAC—or fibrosarcoma—bearer's skin as in mice grafted first with normal skin. Serum from C57BL mice bearing EAC afforded a longer survival to skin grafts from normal C57BL and CBA mice, if injected into recipients of the grafts. Prolonged survival of the skin transplants is ascribed to "protective" substance(s) produced in mice with EAC, viz. to hypoxia and anaemia of the skin present in the mice with fibrosarcoma.*

INTRODUCTION

SKIN TRANSPLANTS from donors with malignant disease may show altered survival as compared with the skin from normal donors. In syngeneic hosts they may undergo rejection [1–7], although this does not happen regularly [8, 9], whereas in allogeneic recipients such grafts exhibit either shortened or, more commonly, prolonged survival. Thus, skin from patients with malignancies survived longer than the skin of normal individuals when grafted onto normal recipients [10] or cancer patients [11], and the same occurred if the skin from animals with transplanted, induced or spontaneous tumours was grafted onto allogeneic recipients [3, 12–17]. The survival of skin allografts from mice with Ehrlich ascites carcinoma (EAC) depended on the size of the tumour in the donor, and was either shorter or longer as compared with the survival of normal skin [3]. Similar variations have been observed by Kaliss and Sutter [15]. The factors that modify the survival of skin grafts from donors with tumours are, however, still obscure.

In these experiments we intended to investigate the following questions:

(*a*) Do the skin grafts from mice bearing methylcholanthrene-induced fibrosarcoma or EAC have a modified survival in allogeneic recipients?

(*b*) If they do, is the survival of the graft correlated with the length of time that the tumour has resided in the skin donor?

(*c*) What may be the mechanism(s) that modify the survival?

MATERIAL AND METHODS

Mice

Donors of the skin were C57BL/H (H—2^b) mice bearing fibrosarcoma or EAC, or normal

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†Permanent address: Institute of Immunology, Rockefellerova 2, Zagreb, Croatia, Yugoslavia.

CBA/H (H—2^k) mice. The recipients were normal mice of A/H (H—2^a) strain. Donors and recipients were males, 3–5 months old.

Tumour

The fibrosarcoma was induced in a C57BL male mouse by a single subcutaneous injection of 1 mg of 20-methylcholanthrene suspended in 0·5 ml of olive oil. Cells of the first and the second generation of this tumour were stored in liquid air. The experiments were performed with tumours of the third generation.

EAC has been maintained in C57BL mice by passage of 0·5 ml of peritoneal fluid at 10–15 day intervals.

Prospective donors of the skin were inoculated subcutaneously with 6×10^3 viable cells of the fibrosarcoma, or intraperitoneally with 10^6 viable cells of the EAC.

Skin grafting

The standard method used in this laboratory [18] was modified in that the sutures were not applied.

Collection of sera

The blood from a particular group of mice (normal or bearing the tumour) was collected by cutting, under ether anaesthesia, the jugular veins, pooled, and kept for 3–4 hr at room temperature. The serum was separated from the cloth by centrifugation.

Haematology

Erythrocytes were counted in blood samples drawn from the tail veins.

Statistics

There were 12–14 recipients in each experimental group. The Student's *t*-test was used for statistical evaluation of the results.

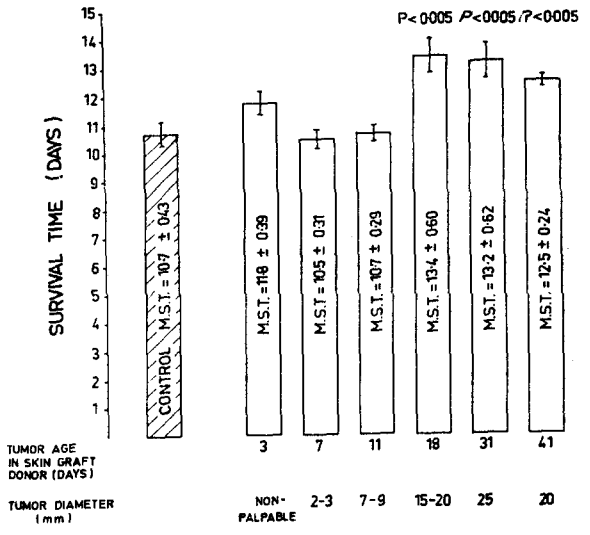

Fig. 1. Mean survival times (M.S.T.) of skin grafts transplanted from C57BL mice bearing fibrosarcoma of different sizes to normal A mice. Control: A mice grafted with the skin from normal C57BL donors. Vertical bars represent S.E. of the mean.

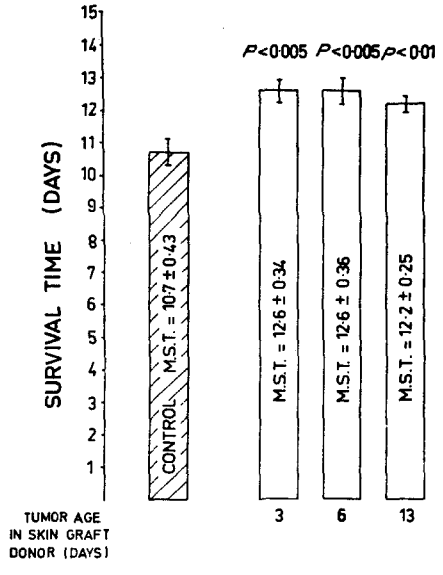

Fig. 2. Mean survival times (M.S.T.) of skin grafts transplanted from C57BL mice bearing EAC to normal A mice. Control: A mice grafted with the skin from normal C57BL donors. Vertical bars represent S.E. of the mean.

RESULTS

The skin from C57BL mice having born either the fibrosarcoma or EAC for different intervals, was transplanted onto untreated A mice. Survival times of these grafts are listed in Figs. 1 and 2. There was no significant difference between the survival of the skin from normal C57BL donors and from those having born the fibrosarcoma for up to 11 days (diameter about 9 mm). However, if the donor had had a larger tumour, grafts of its skin survived significantly longer ($P<0·005$). The skin from donors with EAC survived longer than that from normal C57BL mice, even if the tumour had grown in the skin donor for only 3 days (Fig. 2).

It was further investigated whether skin grafts from C57BL mice bearing either tumour might be less susceptible to rejection than the normal skin when transplanted onto A recipients sensitized by the skin from normal C57BL donors rejected a month before. Figure 3 contains the results. The skin from donors with

the fibrosarcoma was rejected as rapidly as that from healthy donors, but the grafts from mice with EAC did survive significantly longer ($P < 0{\cdot}005$), suggesting that this skin was indeed less susceptible to allograft reaction.

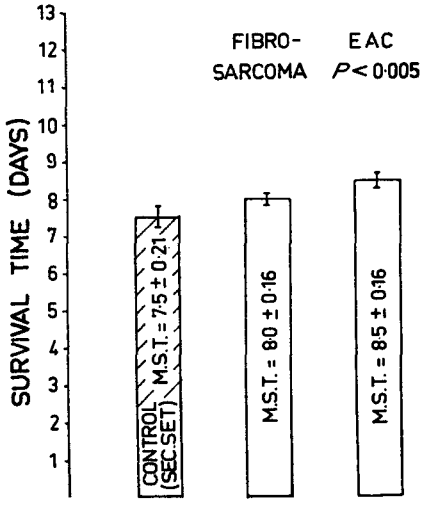

Fig. 3. Mean survival times (M.S.T.) of skin grafts from C57BL mice bearing fibrosarcoma of EAC transplanted to A mice that have previously rejected grafts of normal C57BL skin. Control: both the first and the second grafts were from normal C57BL mice. Vertical bars represent S.E. of the mean.

In the next experiment the ability of the skin from animals with tumours to sensitize towards normal skin was determined. Strain A recipients that had rejected, with a delay, the skin from C57BL donors with tumours, received skin from normal C57BL mice. These second grafts, applied about a month after rejection of the first ones, were destroyed as promptly as the grafts placed onto mice sensitized with normal C57BL skin (Fig. 4). Obviously, the skin of mice with either tumour could effectively sensitize the recipient towards the allografts of normal skin.

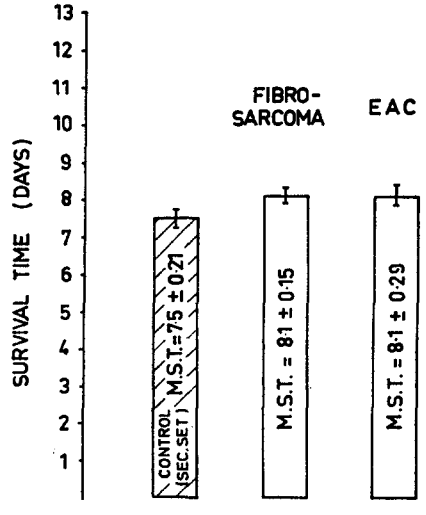

Fig. 4. Mean survival times (M.S.T.) of second-set skin grafts from normal C57BL donors in A recipients that have rejected with a delay the skin grafts from C57BL mice bearing fibrosarcoma or EAC. Control: the first grafts were from normal C57BL mice. Vertical bars represent S.E. of the mean.

In the course of neoplastic disease auto-antibodies against normal tissues may appear [19]. If specific for the skin, they could protect the grafted skin from the immune reaction of an allogeneic recipient (enhancement). To determine whether this was the case, A mice were injected intravenously with 0·3 ml of the serum from C57BL mice with fibrosarcoma or

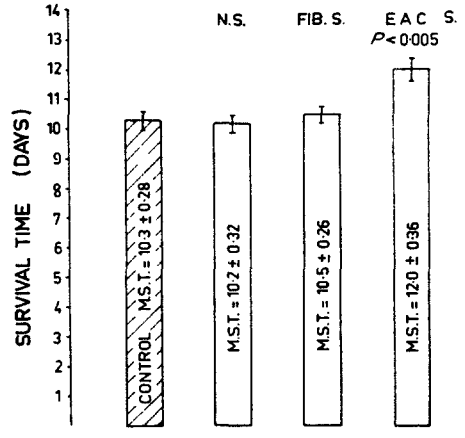

Fig. 5. Mean survival times (M.S.T.) of skin grafts transplanted from normal C57BL donors to normal A mice (Control) or to A mice injected with normal C57BL serum (N.S.), serum from C57BL mice bearing fibrosarcoma (FIB.S.), or serum from mice with EAC (EAC S.). Vertical bars represent S.E. of the mean.

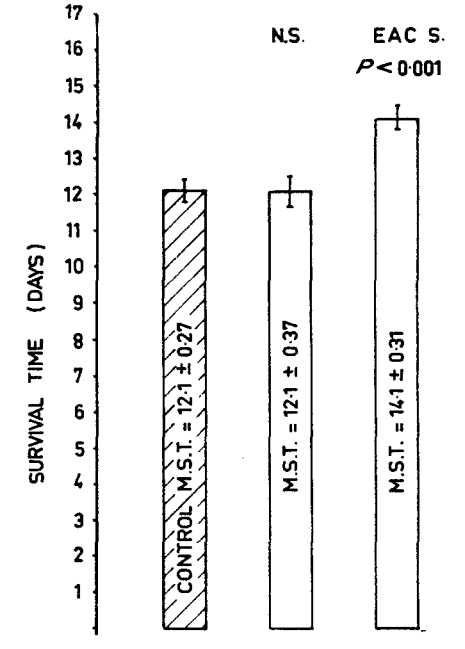

Fig. 6. Mean survival times (M.S.T.) of skin grafts transplanted from normal CBA mice to normal A mice (Control) or to A mice injected with normal C57BL serum (N.S.) or with the serum from C57BL mice bearing EAC (EAC S.). Vertical bars represent S.E. of the mean.

EAC. The serum was applied 2–3 hr before grafting the skin from normal C57BL donors. The results in Fig. 5 show that the serum of healthy mice and that of mice with the fibrosarcoma had no effect on the allograft survival. On the other hand, the serum from mice bearing EAC extended the survival of the normal C57BL skin significantly ($P < 0{\cdot}005$).

Since only the serum from mice with EAC was effective, the specificity of this effect was further tested. For that purpose, 0·3 ml of the serum was given to A mice before these were grafted with CBA skin. Because the serum prolonged the survival of CBA grafts as effectively as that of C57BL grafts, its effect is probably non-specific (Fig. 6).

Skin grafts from mice exposed to hypoxia may survive in normal allogeneic recipients longer than the grafts from normally oxygenated donors [20]. Since the normal tissue of animals with tumours may be hypoxic as a consequence of anaemia which often develops during growth of the tumour, erythrocyte counts were determined in the peripheral blood of C57BL mice carrying fibrosarcoma. The number of erythrocytes started to decline when the tumour had reached about 10 mm in diameter (Fig. 7).

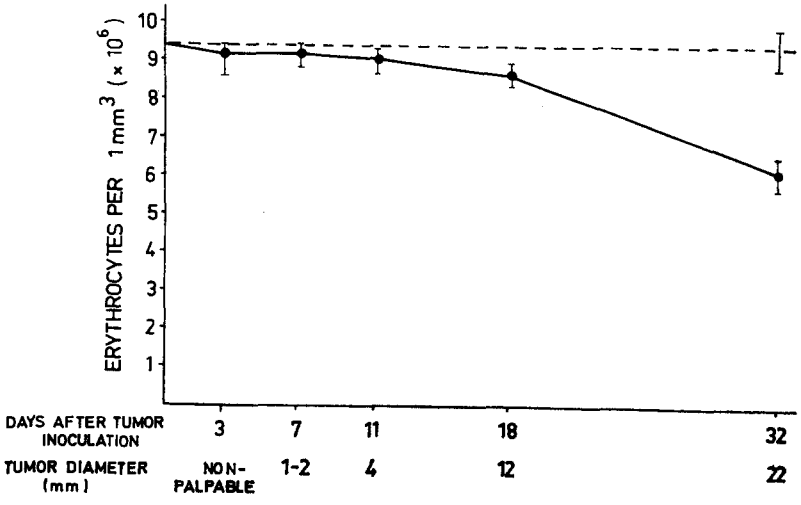

Fig. 7. Mean erythrocyte counts in the peripheral blood of normal (- - - -) and fibrosarcoma-bearing (———) C57BL mice. Vertical bars represent S.E. of the mean.

DISCUSSION

Both the fibrosarcoma and the Ehrlich ascites carcinoma caused the skin of their hosts to survive longer in allogeneic recipients than the skin grafts from healthy mice. The fibrosarcoma influenced the survival of the skin only if larger than 10 mm in diameter, but EAC was effective in this respect regardless of its size. In both cases, however, the delay in the rejection was rather limited. This agrees with the observations by other investigators [13–15].

There is no definite explanation for the prolonged survival of skin allografts from tumour-bearing donors. Decreased concentration of antigens (antigenicity) of the skin, reduced capacity of skin grafts to elicit an immune response (reduced immunogenicity) and immunological enhancement have been proposed as possible mechanisms [13–17].

Mice that rejected skin grafts from donors bearing either fibrosarcoma or EAC destroyed second-set grafts from healthy C57BL donors in an accelerated manner. This suggests that immunogenicity of the skin from mice with the tumours was not impaired, i.e. this skin could efficiently sensitize the recipients towards normal skin. Since the grafts from EAC-bearing mice (but not from mice with fibrosarcoma) survived longer even in sensitized recipients, a reduced antigenicity could be considered as another possible reason. The fact that immunogenicity was not impaired (see above) argues against this possibility.

In tumour hosts, normal tissues including the skin might incorporate antigens specific for the tumour. These would then elicit enhancing antibodies protecting the skin (as well as the tumour) from rejection. In fact, Wexler *et al.* have recently reported that incubation of grafts from normal C57BL skin in the serum of C57BL mice having lymphosarcoma, or the injection of this serum into BALB/c mice to

be grafted, prolonged the survival of the transplanted skin [16, 17]. In our experiments, the serum from mice with EAC afforded a longer survival to the grafts of skin from normal C57BL donors, but delayed the rejection of CBA skin as well, showing that the effect was not specific for C57BL grafts. We assume, therefore, that the growing EAC causes some substances to be released into ascitic fluid and into circulation. These agents acting by immunosuppression [21] or through some other mechanism, could attach themselves to the normal skin and thus prolong its survival in allogeneic hosts, even in the sensitized ones.

This assumption, however, can not be extended to explain the behaviour of the skin from mice with fibrosarcoma. Here, the slight anaemia found in donors of the skin remains as a possibility, since this anaemia could have made the skin hypoxic, whereby it became less susceptible to rejection [20]. It should be recalled that the anaemia appeared only after the tumour had enlarged over 10 mm in diameter, and it was only then that the fibrosarcoma started to influence the survival of the skin grafts.

We conclude, therefore, that both tumours used in this study can prolong the survival of the skin of their hosts in allogeneic recipients. The mechanism of this effect, however, may be quite different.

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Investigations on the Possibility of a Thermic Tumour Therapy—I.

Short-Wave Treatment of a Transplanted Isologous Mouse Mammary Carcinoma*†‡

KRISTIAN OVERGAARD and JENS OVERGAARD
Cancer Research Institute, Danish Cancer Society, Radiumstationen, DK 8000 *Aarhus C, Denmark*

Abstract—*The existing literature on studies of the inhibitory effect of moderate heat doses on malignant tumours is briefly reviewed.*

An experimental technique of heat treatment of mouse tumours in vivo *under accurate control of the intratumoural temperature is described.*

In experiments with an isologous transplantable mouse mammary carcinoma, controlled application of a moderate heat dose led, in many cases, to a permanent cure of the transplanted tumour without causing any damage to the surrounding normal tissue.

The necessary heat doses in the temperature range of 41·5 *to* 43·5°C *are worked out revealing a definite relationship between temperature and exposure time.*

The heat treatment induces distinct histological changes in the tumour cells, whereas it does not cause any damage to the stromal and vascular cells in the tumour, or to the surrounding normal tissue.

Immediately after the heat application, definite changes were revealed in the mitochondria and lysosomes of the tumour cells. These changes increased in intensity with the size of the heat dose and become more pronounced within a few hours or days. Within the first few hours, changes in the nuclei of the tumour cells and in chromosomal and nucleolar chromatin developed, with some variation in the individual cells. 24 *hr after the application of a curative dose, all tumour cells showed severe injury. After smaller doses the reaction to the heat was less intense, with variations in the individual cells, and several tumour cells did not show any signs of lethal injury.*

Autolytic disintegration of the heat-damaged tumour cells occurs very rapidly. The connective tissue of the stroma increases markedly in volume, and a scar forms.

The histological examination did not reveal all details of the process, but in the light of biochemical observations, it is reasonable to assume that the direct effect of heat is due to an elective activation of the acid hydrolases localized in the lysosomes of the tumour cells.

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‡The experimental and histological investigations were carried out by Kr. O., the histochemical investigations by J. O.

INTRODUCTION AND HISTORY

DURING the past century it has repeatedly been claimed that heat may exert an inhibitory effect on a malignant tumour, or even cure it.

A considerable number of clinical reports have been published on permanent cures or protracted improvements of confirmed malignant tumours after disease with a high-grade

fever [1–9], and Cooley's tumour treatment may somehow be associated with heat, as the successful results were mostly obtained in patients with a high fever of long duration [10–12]. Moreover, in recent years, Cavaliere *et al.* [13] and Mondovi *et al.* [14] have reported a number of cures of human tumours treated with local application of heat.

Numerous experimental investigations of the relation between high temperature and the deletion of a tumour have given some hints, but no clarification.

In 1903, Jensen [15] found that transplantable sarcoma tissue tolerated a heat dose of 46°C for 5 min (46°C/5 min), but that it lost its transplantability when heated to 47°C/5 min.

Similar experiments have shown that almost all investigated transplantable tumours in tissue or culture are influenced by heat, and it has been demonstrated that lower temperatures of about 41·5–42°C may destroy the viability of the tumour tissue if the exposure time is sufficiently long, i.e. up to 15 to 20 hr [16–39].

The values reported vary to some extent, but nevertheless show an acceptable agreement in view of the variations in the experimental techniques in which the ability to "take", the growth of the tissue culture, the mitotic index or the staining susceptibility are used as criteria for the viability of the tumour tissue.

Heat tolerance is probably somewhat higher in normal tissue than in tumour cells. The classical Cohnheim experiment [40] in a rabbit ear showed a tolerance of 45°C/30 min or 49°C/6–7 min, and other tissues have also shown a relatively high tolerance [41, 42].

The specific testicular epithelium is an exception showing reaction at a temperature as low as 40–41°C [43–48].

Cultures of normal tissue (mostly relatively primitive) are fairly insensitive to heat and tolerate temperatures of 42–43°C continuously, and somewhat higher temperatures for a short period [29, 34, 35, 41, 42, 49–58]. At the higher temperature general retardation of growth and some morphological changes may occur [51–54, 56, 58].

This difference in heat sensitivity was demonstrated very clearly by Chen and Heidelberger [59] in experiments on induced malignant cell transformation: the higher heat sensitivity seems to be an integral part of the characteristics of the malignant cell.

Tumour tissue shows a distinct reduction in aerobic glycolysis on a moderate increase in temperature, while anaerobic glycolysis diminishes at 42–43°C, accompanied by irreparable cell damage [13, 60–64].

On the other hand, aerobic glycolysis in normal parenchymatous tissue (e.g. regenerating liver tissue) does not react to heat doses of that size [13, 62–64].

Heat exposures within the same range reduce the synthesis of nucleic acids and proteins in tumour cells [64, 65], while the growth of normal cells is not impaired.

The effect of this growth-inhibiting process depends to a great extent on the growth rate of the tumour cells [66] and may be intensified by a number of added drugs [37, 67, 68].

Biochemical investigations on tissue homogenates suggest that the heat action is somehow associated with the lysosomal function, but possibly is not primary here [63, 69].

Many experiments with heat treatment of transplanted tumours suggest that the possibility of cure exists, but the results vary widely because of the technical difficulties encountered.

A sufficient generalized heating of the tumour-bearing animal in a water bath, hot chamber or high-frequency (h.f.) field is very difficult on account of the general heat reaction, but in some cases a beneficial effect has been obtained [28, 70–76], and this treatment is an integral part of the attempts made by Ardenne at a multi-stage cancer therapy [77].

Local heating of the tumour and its surroundings by external heat application (water bath, microwave, ultra-sound) is possible only in superficial tumours, as vascular cooling makes an action on the deeper parts impossible. Nevertheless, some effects with such approaches have been reported [32, 33, 78–93].

In many experiments, local treatment of the tumour with h.f. currents has resulted in permanent cures of transplanted tumours without serious damage to the surrounding tissue [23, 60, 94–106].

In most of these experiments, no attempts were made to measure the intratumoural temperature during the treatment, but measurements after the treatment showed a local or a generalized increase in the body temperature in several cases. In some experiments, temperature measurements during the treatment were attempted, but the methods used were unreliable and misleading [60, 106].

Insufficient understanding of technical manifestations has led some investigators to presume that a narrow range of h.f. currents (88·2 to 93·7 MHz) might have a specific athermic tumour-damaging effect [107–112], but further investigations unequivocally indicate that heat is the only biological factor acting on the tumour tissue in question, and that the great effect of some wave lengths of h.f. currents is due to

some unrecognized technical factor [91, 100, 104, 113–116].

PRESENT STATUS AND PURPOSE

Several observations—both clinical and experimental—indicate that a moderate influence of heat may inhibit the growth of, or completely destroy, malignant tumour tissue, but although a considerable number of cures of transplanted tumours are on record, we actually know very little about the dose of heat which is necessary to produce such an effect. Our knowledge of the histological reactions in heat-treated tumour tissue is very sparse, and the mechanism of action of the heat applied is still unclarified.

As, nevertheless, the question may be of therapeutic interest, we have carried out some experiments in an attempt to clarify the following problems.

(1) Will local heat application destroy a transplanted tumour without damaging the surrounding normal tissue?

(2) In what ranges of temperatures and exposure times will such an effect be possible?

(3) How are the histological and histochemical reactions of the tumour tissue to such doses?

(4) Will the findings give a probable explanation of the mechanism of action of the heat?

MATERIAL AND METHODS

C_3H mice were inoculated subcutaneously in the flank with an anaplastic mammary carcinoma HB, which has originated in the C_3H strain of the Institute. Untreated animals with a certain tumour growth all died with a large local tumour within 3–6 weeks. The "take" and growth rates of the tumour were apparently independent of the sex of the animals. Metastases were rare.

About 1200 mice with tumours measuring $5 \times 5 \times 6$–8 mm were anaesthetized with an intraperitoneal injection of Nembutal (72 mg/kg) to which was added 1 ml of isotonic glucose solution. They were then treated with local short-wave diathermy (27·12 Mc) using a model generator with an output of 1 W and about 25 V and insulated electrodes of 2–4 cm^2 under strict control of the intratumoural temperature by means of a special h.f.-neutral thermocouple (Fig. 1). By automatic regulation of the generator output it was possible to maintain any desired temperature continuously with a variation of about 0·1°C. The heat dose was designated as a fraction, the numerator being the temperature in °C and the denominator the exposure time in minutes.

The starting temperature in the tumour was about 35 to 36°C. The time from the start of the heating until the planned temperature was reached (mostly 3 to 5 min) was noted, but not included in the heat dose. By this method it was possible to obtain a temperature in the central part of the tumour of up to 46 to 47°C, apparently without damaging the skin or the underlying tissue.

Fig. 2. H.f.-balanced thermocouple. Air-filled lumen of glass tube: 1 mm. Copper and constantan wire, about 0·05 mm.

Critique of the technique

The technique used is far from ideal. For technical reasons, both the electrodes and the thermocouple must be relatively large in relation to the small object of treatment, and there is a certain risk of incorrect placement. Moreover, the h.f. currents heat different types of tissue unevenly, and differences in the cooling effect of the vascularization may also play a certain role.

There are thus a number of uncontrollable factors, all tending to give less heating in some parts of the tumour than registered.

A relatively well-conducting tumour situated in loose connective tissue can be heated rather uniformly if it is of regular shape, but in an irregular tumour with delicate ramifications in fatty tissue the temperature will be higher in the central part than in the ramifications.

As the intensity of the short-wave field is high around metals, but falls around an air bubble or non-conducting material, it is necessary to use a thermocouple balanced for the h.f. field, as temperature measurements will otherwise be unreliable [117]. The type used (Fig. 2) will afford an accurate value, but only at the soldering point, and there may be an indeterminable difference in temperature between various parts of the tumour.

RESULTS OF THE TREATMENT

Of the animals studied, 173 succumbed during anaesthesia and treatment, while 710 which survived for at least 10 days were used

in the evaluation of the curative effect of the various doses applied. All the mice were exposed to only one heat treatment.

Initial experiments had shown that heat doses of 43°C/45 min resulted in complete disappearance of the tumour in 20% of the treated animals. As a moderate increase in the dose—as regards temperature and exposure time—did not change the results appreciably, we found it likely that the method, on account of the above mentioned factors of uncertainty with which the technique was beset, had its practical limit at this level, and the value of the lowest curative exposure time in the temperature range between 41·5°C and 43·5°C was worked out without variation in the procedure. The results appear in Table 1.

some cases, the tumour infiltrated the skin, which, together with the tumour, formed a necrotic crust within a few days, which remained for 2 or 3 weeks and gradually exfoliated, leaving a small hairless scar. In a few cases, discoloration of the hairs in the treated region occurred, and local alopecia developed in two animals.

The cured animals were normally observed for at least 6 months. Evidence of local recurrence was observed only in one animal, after a tumour-free period of 3 months.

If the technical conditions had failed, the initial reaction would have been the same, but within 2 or 5 days the growth would have been resumed—usually at the pole of the tumour—and be progressing as if no treatment had been given.

Table 1. *Curative effect in* 710 *mice surviving for at least* 10 *days after local heat treatment. The total number of surviving mice, the number of cures obtained, and the percentage of cures are shown for each dose.*

Heat dose (°C)	12 min	15 min	20 min	30 min	45 min	60 min	90 min	120 min	180 min	240 min
41·5						27 0 0%		32 0 0%	14 1 7%	20 8 40%
42·0				20 0 0%		18 1 6%	16 1 6%	18 4 22%		
42·5°				15 1 6%	17 3 18%	26 2 8%	34 8 23%			
43·0		15 0 0%		18 2 11%	40 8 20%	63 16 25%				
43·5	8 0 0%		35 1 3%	103 9 9%	9 2 22%	162 41 26%				

Clinical course

Immediately after the treatment, tumours treated with a single adequate heat dose showed a slight increase in size, but already on the next day the tumour had decreased and become slightly firmer in consistency. During the following days, the decrease in size and the increase in consistency continued; so that the tumour had completely disappeared after the lapse of 2 to 3 weeks, usually without leaving any visible scar. In most cases, the skin covering the tumour showed no alterations at all during this process, and no ulceration occurred. In

In the cases treated with non-curative doses we often found some alterations in the consistency of the tumour, and the growth rate was occasionally reduced for some days, but in all cases the growth of the tumour progressed. All animals showing renewed growth died with a large tumour within 6 weeks.

In the experiments, 149 tumour-bearing mice were selected as controls and given no treatment. Another 48 mice were given sham treatment with anaesthesia and introduction of a thermocouple placed in dummy electrodes without any electrical tension for 60 min. All

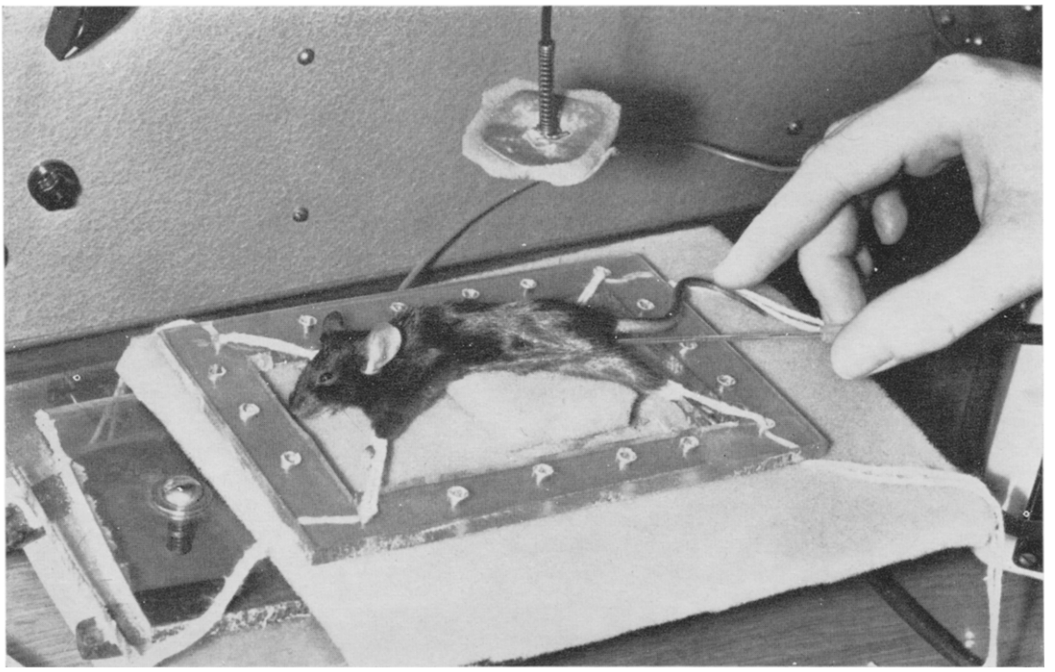

Fig. 1. *Placement of tumour-bearing mouse between insulated electrodes. Introduction of thermocouple.*

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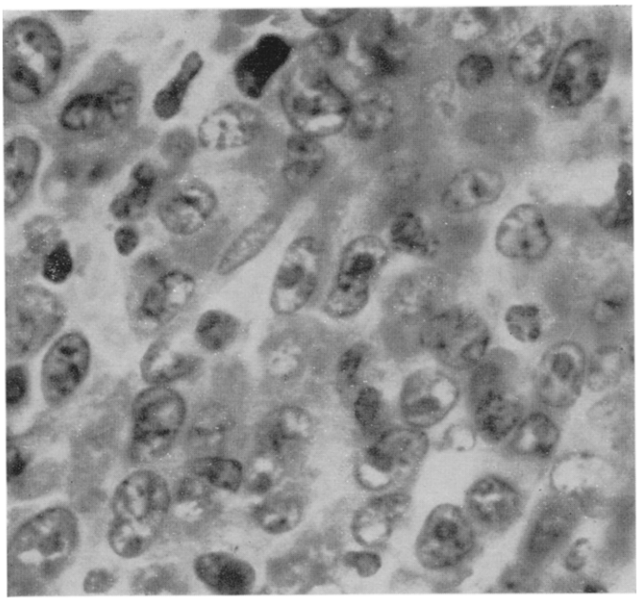

Fig. 3. Untreated tumour tissue. Closely packed tumour cells without distinct boundaries. The cytoplasm almost unstained. Large vesicular nuclei with well-defined central nucleolus. Stroma sparse, a single spindle-formed nucleus visible. Haematoxylin-eosin staining. 1500 × .

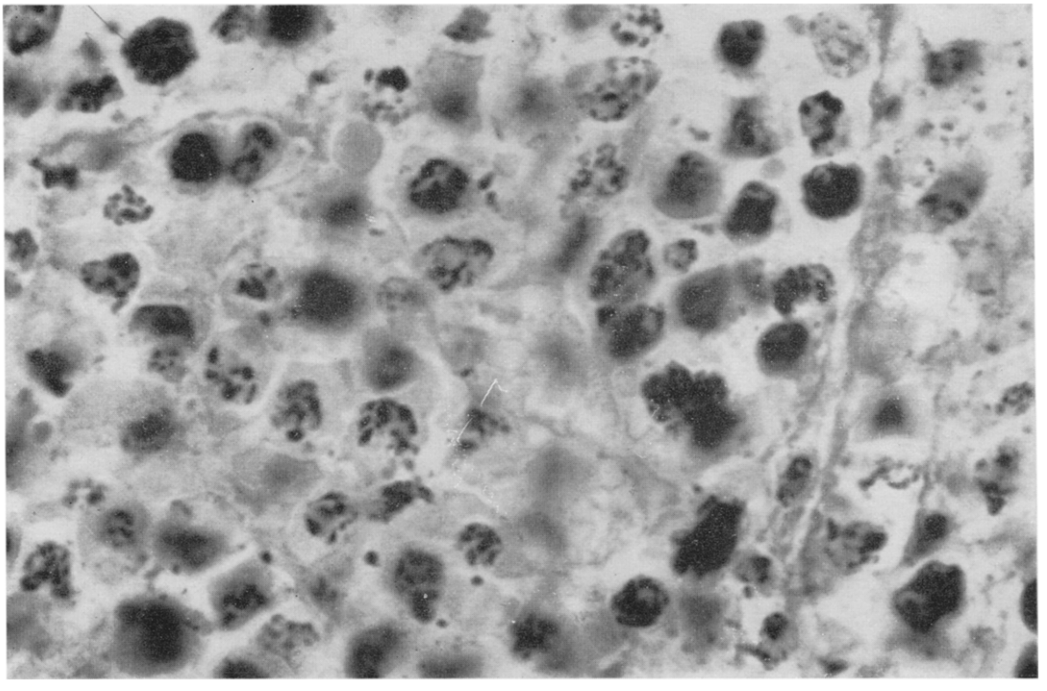

Fig. 4. Three hours after treatment with 43°C/60 *min. Most of the tumour cells show moderately defined boundaries. The cytoplasm is moderately stained. In some cells, the nuclear boundary is well-defined and the nucleus angulated. The chromatin is collected in small clumps. In some cells, the nuclear boundary is ill-defined and the chromatin is collected in small, intensely stained clumps. In most cells, the nucleolus is not discernible. Haematoxylin–eosin staining.* 1500 × .

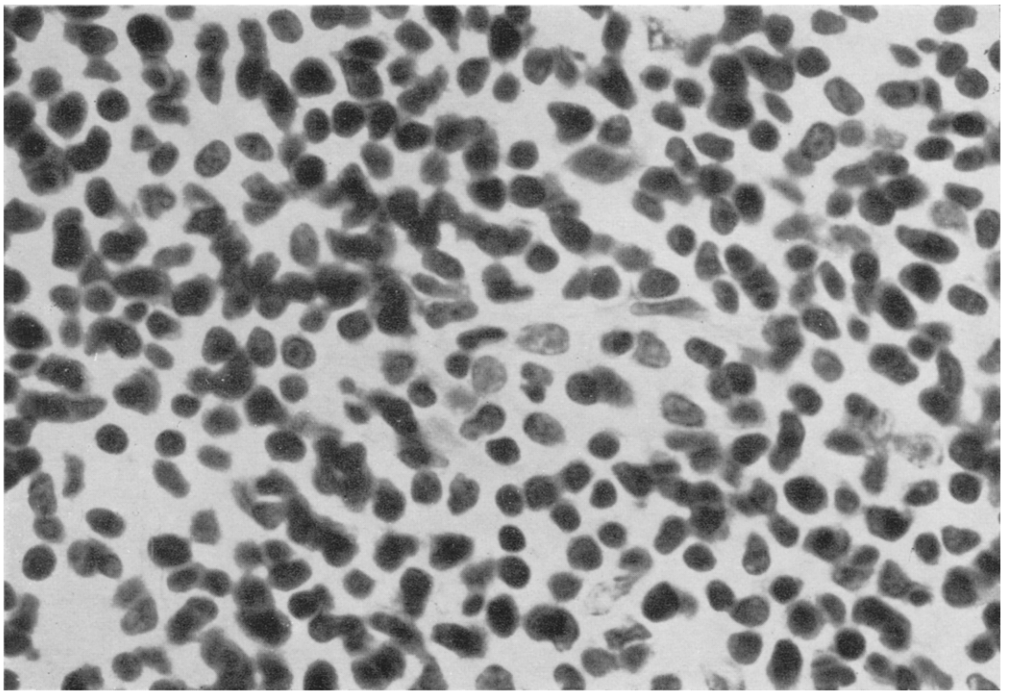

Fig. 5. *Twenty-four hours after treatment with* 43·5°C/60 *min. Markedly shrunken tumour cells. The small, rounded nuclei are intensely stained. The cytoplasm has been reduced to a narrow halo. The stromal and vascular cells are undamaged. Haematoxylin–eosin staining.* 1500 × .

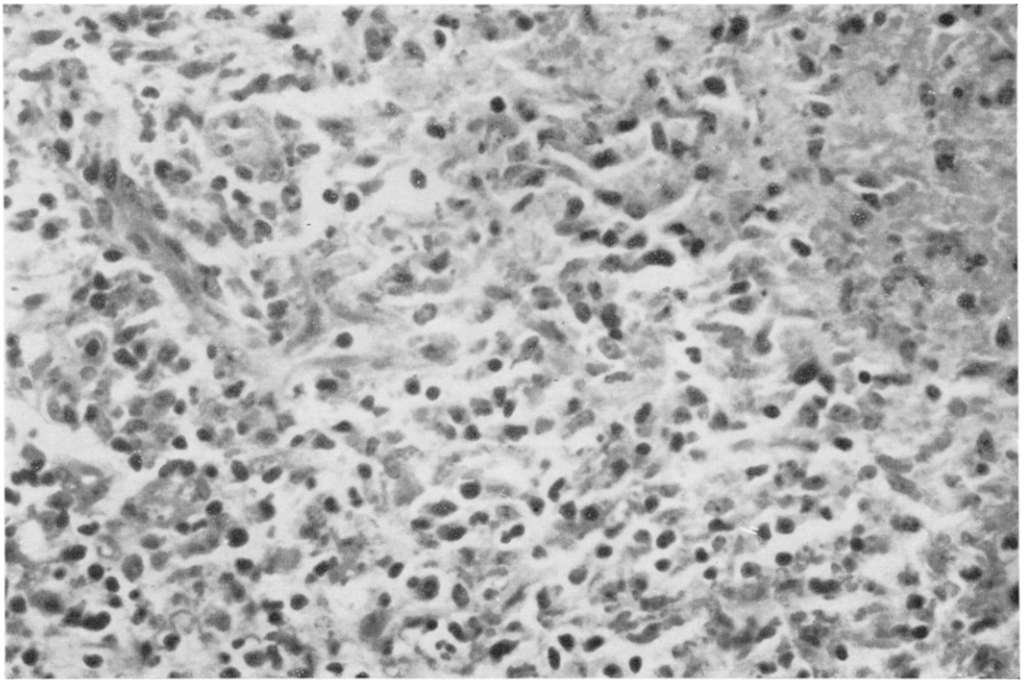

Fig. 6. *Three days after treatment with* 43·5°C/60 *min. Tumour cells intensely shrunk. Moderate karyorrhexia. Stromal connective tissue invading the tumour in fine streaks. Haematoxylin–sirius staining.* 600 × .

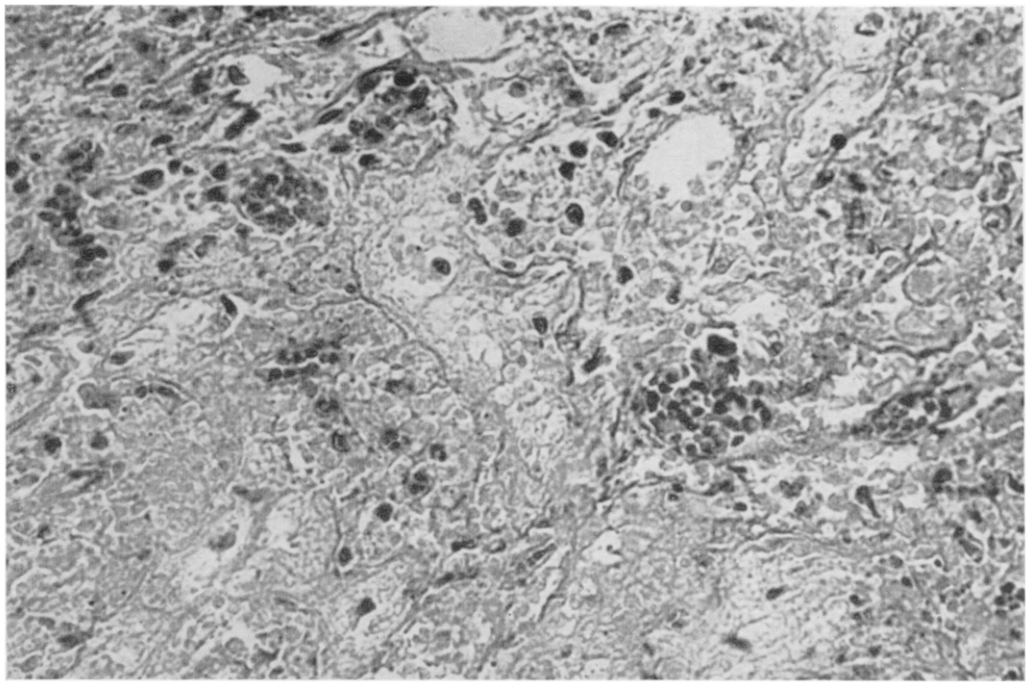

Fig. 7. *Four days after treatment with* 43·5°C/60 *min. Heavy growth of stromal connective tissue isolating small nests of pyknotic tumour nuclei. Haematoxylin–sirius staining.* 400 × .

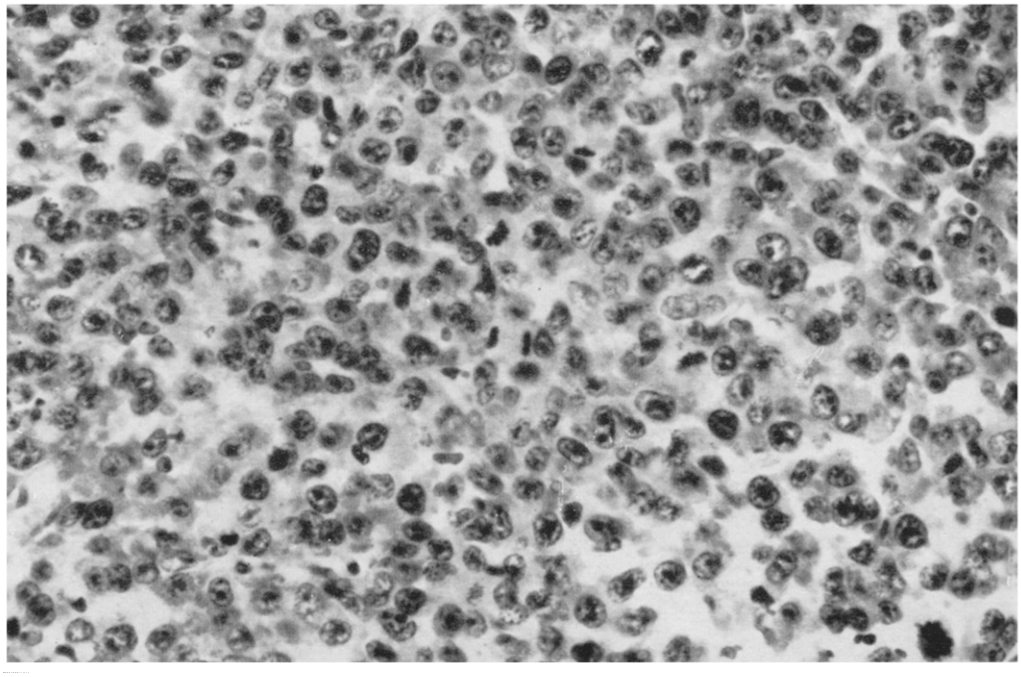

Fig. 8. *Twenty-four hours after treatment with* 41·5°C/60 *min. Tumour cells damaged in variable degrees: conspicuously unaltered cells; slight or moderate chromatin clotting; some markedly shrunken nuclei; cells with "large, dark nuclei"; many irregular "arrested" mitoses. Stromal cells undamaged. Haematoxylin staining.* 600 × .

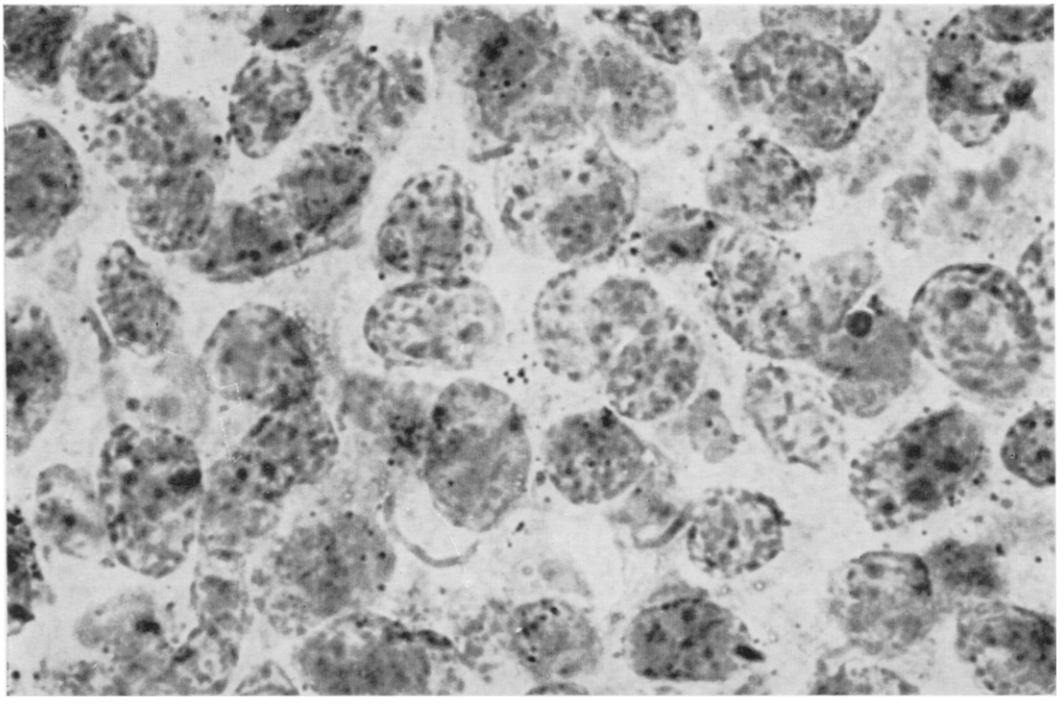

Fig. 9. *Untreated tumour. Uniform structure of all cells with small acid–phosphatase-positive granules. No diffuse staining of the cytoplasm. Acid phosphatase.* 1500 × .

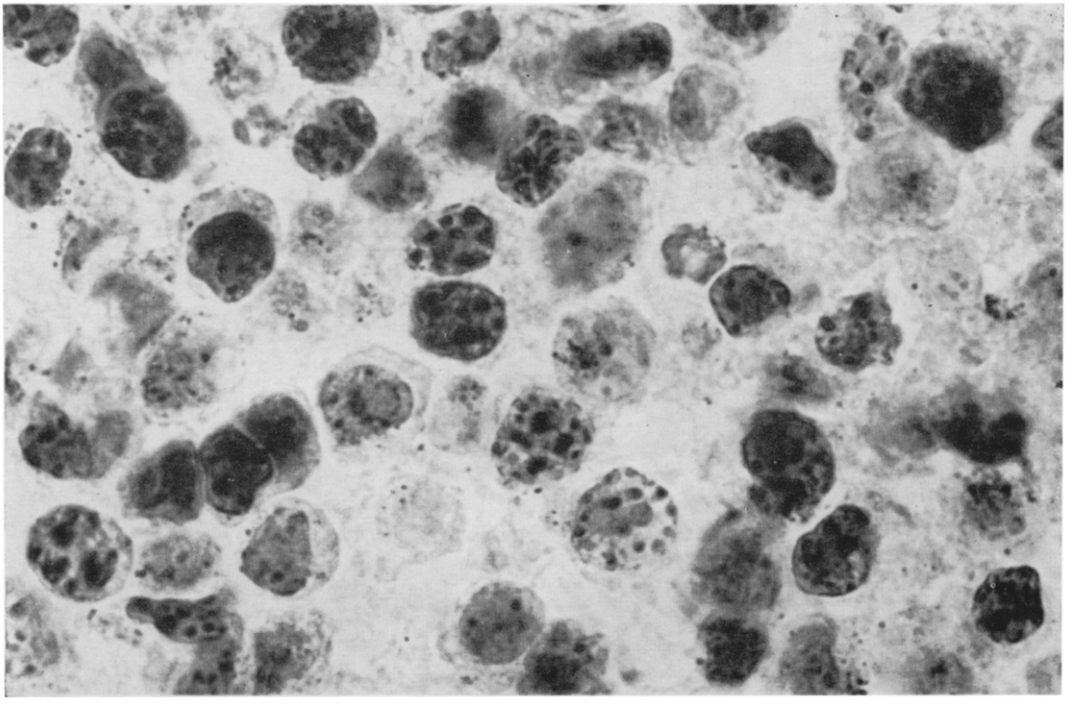

Fig. 10. *Four hours after treatment with* 41°C/60 *min. Granules of variable size (up to* 3μ) *with strong acid phosphatase reaction and, in some cells, diffuse cytoplasmic staining. The cellular structure is still preserved. Acid phosphatase.* 1500 × .

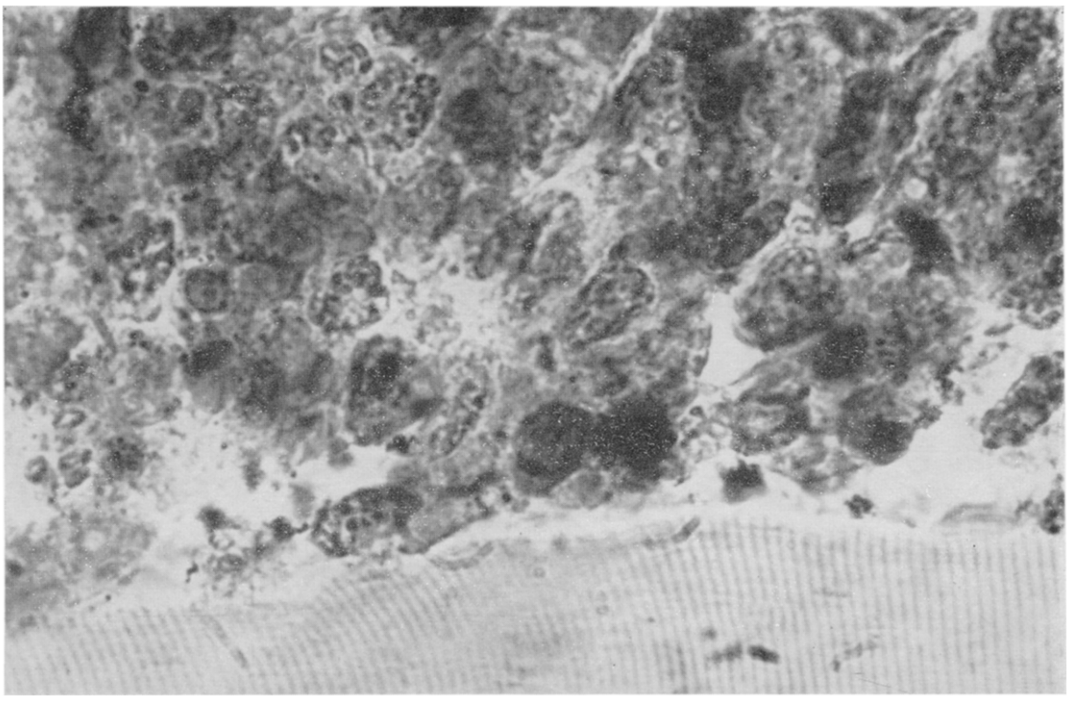

Fig. 11. *Twenty-four hours after treatment with* 43·5°C/60 *min. In all cells a pronounced reaction with large, dark-stained granules and cytoplasmic reaction. Some areas with necrosis. The adjacent muscle without reaction. Acid phosphatase.* 1500 ×.

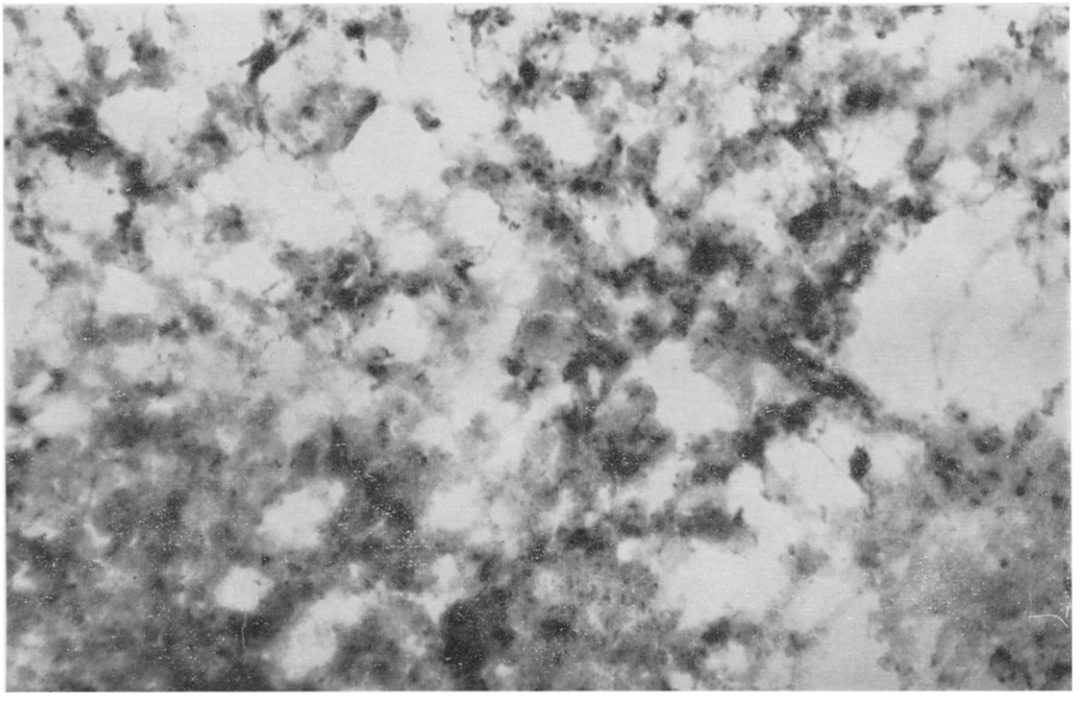

Fig. 12. *Three days after treatment with* 43·5°C/60 *min. The whole tumour is necrotic. There are areas with diffuse positive staining, but in large parts the enzymatic reaction has worn off. Acid phosphatase.* 150 ×.

the control animals died with a large tumour within 6 weeks.

HISTOLOGICAL INVESTIGATION

The curative effect was also studied histologically in tumour tissue which had been exposed to the above-mentioned or somewhat smaller heat doses.

Technique

More than 250 heat-treated tumour-bearing mice were studied. They were killed by occipital luxation, and the tumour tissue was immediately excised and prepared. The tissue was fixed in Helly's fluid, stained with Heidenhain's iron–haematoxylin, Mayer's haematoxylin–eosin, and Haris's haematoxylin–sirius dye for connective tissue. Histochemical assays for acid hydrolases and succinate dehydroxygenase were performed in many cases.

Microscopic examination of the site of the tumour was performed in almost all cured mice when killed or on spontaneous death. A total of more than 300 mice were examined microscopically.

Histological examination of tumour tissue was performed (1) just before heat treatment, (2) immediately on treatment and (3) at varying times after treatment, at first at intervals of a few hours and later at intervals of a few days. As the rate of alterations decreased, the intervals were gradually extended in order to obtain a complete picture of the course of the reaction.

Most of the investigations were carried out on tumours treated with doses within the therapeutic range, but the effect of smaller doses was also studied.

Histological findings

Histological examination of the untreated tumour tissue (Fig. 3) showed closely packed, large, bright cells with indistinct cell membranes and large vesicular nuclei with a sparse, granular chromatin structure and a large central nucleolus. Stromal and vascular tissues were sparse. Even in small tumours there were often well-defined foci of spontaneous necrosis at different stages, mostly easy to distinguish from heat-induced alterations. In the fresh parts a moderate number of mitoses were recognizable, and in the examination of early heat reactions we looked for parts in which mitoses or remnants of mitoses might be present.

Immediately after treatment in the curative range we often found moderate hyperaemia, but the tissue structure showed no conspicuous changes.

During the following 2 to 5 hr the cytoplasm shrank slightly; the cell boundaries became more distinct and the staining more intense. The nucleus became angular, and the nuclear boundary was intensely marked as small chromatin granules settled on or near the inner border of the nuclear membrane. Most of the chromatin had collected in moderate clumps (Fig. 4), more irregularly in the nuclear space, and the nucleolus was often indistinct or had disappeared. During the next few hours the nucleus shrank considerably and the chromatin clumps fused and grew larger. The rate of development of these changes was not uniform in the individual cases, nor in all the malignant cells of the same tumour, but 24 hr later the size of the nucleus had decreased appreciably, and the chromatin had collected in a single irregular, dense, intensely stained clump (Fig. 5). The cytoplasm appeared as a dark edge often containing small vacuoles. These changes could be demonstrated in all tumour cells in the bulk of the tumour.

Only in a few cases did we observe some tumour cells infiltrating the surrounding connective tissue near the pole or border of the tumour, which showed signs of minor damage as mentioned below.

Among the shrunken tumour cells, normal bright nuclei of stromal connective tissue and vascular cells could be seen, and the surrounding normal tissue was unaffected.

No new mitoses were seen, and mitoses in progress grew plump and clotty. Cells at prophase remained unchanged for a relatively long time showing a regular round, uniformly stained nucleus surrounded by cytoplasm of an almost normal appearance, but 24 hr later it was almost impossible to find remnants of any mitoses.

During the next few days, nuclear pyknosis and cell shrinkage progressed, and some karyorrhexis developed (Fig. 6). At the same time, the stromal connective tissue increased, at first in fine streaks, later in coarser streaks isolating large or small nests of shrunken tumour cells (Fig. 7). Some macrophages, but remarkably few other cells were present.

In the course of 8 to 14 days, the tumour tissue disappeared completely and only connective tissue remained; at first it was of a loose texture; later it assumed a fibrous character. After 6 months, this tissue appeared as a fibrous cicatrix, varying from a moderate thickening in the subcutaneous connective tissue to a fibrous mass of the size of a pea.

In cases in which the tumour regressed in spite of a curative dose, the histological picture

was of quite the same character for the first few days, but then tumour growth of common type became visible in some places—mostly at the margins—completely isolated from the tissue destroyed by heat. During the next few days, new growth of tumour tissue dominated the picture, but islands of heat-reacting tumour tissue might be present for some days.

The observations made after considerably smaller doses offered a striking contrast to the histological picture just described. 24 hr after a dose of 41·5°C/60 min (about 1/4 of the curative dose) some cells displayed slight clumping of the chromatin, but in most of the tumour cells no conspicuous changes were observed. Mostly, the tumours showed a very large number of mitoses, almost all irregular and plump metaphases (Fig. 8). At 48 hr, many disintegrated cells at metaphase were present, apparently evidencing arrest of mitosis.

In some zones—mostly near the border of the tumour—a number of cells showed well-defined boundaries. The nucleus was of normal size, but often somewhat angular and rather ill-defined. The nucleus was apparently uniformly dark-stained. A very slight haematoxylin staining revealed a moderate chromatin concretion dispersed in a diffusely stained nuclear sap, distinctly different from the common type of reaction. In most of these cells no traces of the nucleolus were seen, but a vacuole of similar size was often present centrally. The cytoplasm was not conspicuously changed, but vacuoles were often seen. This type of cells could be found in decreasing number 2 or 3 days after the treatment.

In the course of 2 or 3 days the tissue contained many regular mitoses at all phases and showed the character of an actively growing tumour. This new tumour growth was found in all places of the tumour (multicentric growth).

24 hr after a somewhat higher dose (41·5°C/120 min = ½ of the curative dose) most of the tumour cells displayed distinct concretions of the chromatin, but not any pronounced shrinking of the nucleus. In some cells, the nuclear shrinking was very pronounced, but many of the cells did not reveal visible alterations. Cells with "dark nuclei" and irregular metaphases were seen in large numbers. The regeneration of the tumour tissue seen on days 2–3–(4) was of the same type as mentioned above.

With doses just below the curative level (42°C/60 min) the majority of the cells showed distinct chromatin clumping and shrinkage, but some cells with "large dark nuclei" or at metaphase were also seen, and there were even a few, apparently undamaged cells.

New multicentric tumour growth might also be seen here within 3 or 4 days.

HISTOCHEMICAL INVESTIGATIONS

Techniques

1. *Succinate dehydrogenase.* The tissue was cooled to −20°C and cut into sections of a thickness of 7μ, which, without further preparation, were mounted on slides and treated by the method of Pearse for succinate dehydrogenase using MTT [118]. The sections were incubated at 37°C for 60–90 min.

2. *Acid phosphatase.* The tissue was fixed in formolcalcium (10% formalin, 1% calcium chloride, pH 7·0) for 24 hr at 4°C. Stored overnight in 0·4 M sucrose in 0·05 M cacodylate buffer, pH 7·4. Quickly frozen in isopentane at −70°C. Sections, 7μ thick, were cut in a cryostate, dried at room temperature and incubated at 37°C for 30 min in Gomori's medium for acid phosphatase. Composition of the medium and further treatment as described by Barka and Anderson [119].

Histochemical findings

1. *Succinate dehydrogenase.* In the fresh, untreated tumour tissue the Pearse MTT staining always showed a normal uniform size of very small cobalt-containing granules regularly dispersed in the tumour cells. In the adjacent muscle cells the long mitochondria were marked by pearl-string-looking regular rows. Only in the vicinity of necrotic regions were some irregular granules seen.

All heat-treated tumour tissue revealed some variation in the size of the granules. The number and size of the irregular granules increased in proportion to both the heat dose and the time from treatment to examination. In many cases, cobalt-stained vacuoles, 2–3μ (or more) in diameter, indicating severe damage to the mitochondria, were seen 1 or 2 days after the exposure to heat. Even in such cases, a large number of normal granules were visible in the tumour cells. In the adjacent muscle cells no irregularities were observed.

2. *Acid phosphatase.* In untreated tumours all the cells showed a moderate number of small (above 1μ) uniform acid phosphatase-positive granules dispersed evenly in the cytoplasm (Fig. 9). Among the neoplastic cells and especially in the surrounding connective tissue

some macrophages with a more intense acid–phosphatase reaction were seen.

During the first few hours after treatment with small doses (e.g. 41°C/60 min), many of the neoplastic cells, particularly in the peripheral parts of the tumour, showed an increase in the number of acid phosphatase-positive granules, in many cells up to a diameter of 3μ (Fig. 10). In the central part of the tumour no definite changes were seen.

During the following days, the peripheral changes became more pronounced. Some cells contained large clumps of active material with diffuse positive staining of the cytoplasm, and a few showed a strongly positive reaction of the whole cell. However, the greater part of the tumour revealed only a moderate abnormal reaction. The macrophage reaction seemed to increase slightly in intensity, both as regards the number of these cells and the strength of reaction.

Immediately after treatment with curative doses (e.g. 43·5°C/60 min), all the neoplastic cells showed an intense reaction with an increase in the number and size of positive granules, which were heavily stained; after some hours several cells showed a strong reaction all over the protoplasm (Fig. 11).

A few days after the treatment, the tumour revealed a picture of ill-defined areas with intense acid phosphatase activity, and the tumour tissue had become a structureless mass (Fig. 12). In some areas, no staining was seen—presumably because the enzymatic activity here had worn off.

A moderate macrophage reaction was seen. The surrounding muscle and connective tissue did not reveal any evidence of damage.

DISCUSSION

The observation that heat treatment may bring about a number of cures in transplanted animals is in good agreement with several previous investigations. Evidently, in some cases local heat application has resulted in the destruction of both the tumour and the surrounding normal tissue [90, 98, 114], but several authors have reported deletion of the tumour without any visible damage to the normal tissue [23, 32, 33, 60, 94–97, 104, 106]. It should be emphasized that any measurement of the intratumoural temperature during the treatment was either omitted or technically inadequate in all previous experiments, whereas, in view of the technique employed, we find it justified to regard our temperature measurements as fairly reliable. Two commonly quoted authors deserve special mention. First, Westermark [60] using long-wave diathermy measured heat doses which were about 4°C higher than ours. His findings may be due to the use of a relatively thick and long, non-insulated thermocouple, which for physical reasons must necessarily record a considerably elevated temperature. Secondly, Crile [32, 33] transplanted tumours to the poorly vascularized feet of mice and treated the animals with local immersion in hot water. The intratumoural temperature was not measured, but the external doses were in tolerable agreement with our results.

Only in our previous series of investigation of the heat effect on Wood's sarcoma was a similar reliable technique used [99–101]. The results of these investigations are in good agreement with our present findings.

As immunological relations between tumour and host were probably disregarded in many of the previous studies, the reported results as to the cures are only of limited value. As our experiments were performed in animals of a highly inbred strain, and with a tumour which had developed in that strain, immunological reactions resulting in some tumour destruction were reduced to a minimum.

Our results may be divided into three groups.

(1) Curative doses by which 20 to 25% of cures were obtained.

(2) Borderline doses after which the tumour showed a violent reaction and some cures were obtained.

(3) Non-curative doses by which no cures were obtained.

That the rate of cures was almost identical in all the curative series, and that a considerably longer exposure time does not lead to an increase in the number of cures are explained by two factors which are necessary in order to obtain a cure: the use of a biologically curative heat dose, and a technically adequate application of this dose to all parts of the tumour.

As several uncontrollable factors may reduce the heat dose in some parts of the tumour, we must suppose that all curative doses are biologically sufficient, but some physicotechnical factors may be blamed for the failure to apply heat to some parts of the tumour in about 3/4 of the experiments.

In this way, the successful results may be of decisive importance, and the method may be used for fundamental conclusions, but in its present form it cannot be applied for a numerical estimation of the effect of a given dose.

A graphical representation of the results obtained indicate a regular exponential relation between the temperature used and the time of heat exposure required to obtain a curative effect.

We did not obtain cures at doses below 41·5°C/180 min, but as both histological and histochemical examinations revealed some effect at 41°C/60 min, a longer exposure at a lower temperature may be able to bring about a cure. Investigations in that field are in progress.

A few investigators have previously briefly reported some changes observed in heat-treated tumour tissue, viz. hyperaemia, pyknosis, absence of mitoses and rapid disappearance of the tumour cells [13, 23, 82–84, 95, 102]; but in most reports only the word "necrosis" was used in describing the changes.

Only in our previous publication [101] were more specific alterations in the tumour tissue described by Okkels, who emphasized both characteristic nuclear and cytoplasmic changes. In view of the differences between the respective tumours and our augmented histological knowledge, our present findings are consistent with previous observations.

The histological appearance after heat treatment is specific. It differs conclusively from the necrotic and necrobiotic processes commonly seen in tumour tissue. These are predominantly caused by failing nourishment and are evidenced by coagulation necrosis with some chromatolysis and terminal chromatorrhexia, even though incipient pyknosis may be present in the boundary zone to living tissue, and the necrotic processes occur only in parts where the tumour growth has been arrested. In addition, spontaneous necrosis involves both the specific tumour tissue and its supporting stroma and vascular tissues.

On the other hand, the heat-provoked alterations affect both rapidly and slowly proliferating tumour tissue, and the alterations are electively localized in the tumour cells, whereas the intermingled stroma and vascular cells are not affected.

Very rapid new growth of connective tissue and simultaneous resorption of the tumour cells are also characteristic of the reaction and differ strongly from the slow growth of connective tissue and resorption which may occur locally around a spontaneous necrosis.

It is also a characteristic feature that in tumour tissue exposed to a heat dose within the curative range, the histological appearance is quite uniform after the lapse of 24 hr, and apparently it does not change even if the dose is doubled

The fine details cannot be estimated by microscopy, but it is likely that, in the nucleus, both the chromosomal DNA and the nucleolar RNA are damaged, and that, in the cytoplasm, both the mitochondrial and the lysosomal structures are severely affected.

In all specimens treated with curative doses, all the visible tumour cells were clearly affected, and not until 2 or 3 days later was it possible to find new tumour growth in limited areas in the unsuccessful cases.

Specimens treated with smaller doses showed cells of apparently different grades of affection, some of them almost undamaged, in complete mixture, and there may be signs of new growth in many places in the visual field. Heat-provoked mitotic abnormalities of the appearance observed are reported to occur in cultures of normal and malignant tissue [30, 54, 120]. Apparently, the occurrence of cells with "large, dark nuclei" has not been observed before; they may correspond to the morphologically changed He–La cells, occasionally showing nuclear vacuoles, mentioned by Selawry [30], and the abnormalities may somehow be related to the heat-provoked nucleolar alterations described by Simard *et al.* [121, 122] and Love *et al.* [123]. Areas showing complete destruction of the tumour tissue were not observed.

After doses slightly below the curative level, the changes are less pronounced; some of the nuclear abnormalities are relatively slight, and new multicentric tumour growth can nearly always be demonstrated after the lapse of 2 or 3 days.

In view of the technical difficulties involved in the application of the heat dose, it is likely that similar slight changes may occur in the periphery of a tumour which has otherwise received a curative dose. However, as we demonstrated such changes only in very few cases, it is reasonable to assume that such foci are very small. With this reservation, it may be said that the histological findings show good agreement with the gross curative effect observed.

Just as the histological investigation displayed highly peculiar findings, also the acid phosphatase investigations showed pronounced and distinctive alterations.

The lysosomes in the untreated tumour cells are, both in number and activity, of a degree which may be a reflection of the physiological destruction and the regenerative processes in the tumour cell, whereas the very intense reaction in the treated tumour cells may have a different explanation.

Previous biochemical findings, reported by de Duve and his co-workers on the activation of isolated lysosomes in liver cells, show that an increasing temperature and augmented acidity accelerate the reaction, and that the same factors intensify the actions of the released acid hydrolases [124, 125].

As, on account of more intense anaerobic glycolysis, malignant tumour tissue possesses a higher acidity than normal tissue [126, 127], we find it reasonable to assume that a similar activation may be released *in vivo* by the induced heat as the internal pH in the tumour cells may be of a value where we, according to Appelman and de Duve [125], may expect a distinct (but not maximal) lysosomal activation at the temperature in question.

In the surrounding normal cells where the acidity is significantly lower, the possibility of such an activation will be considerably less.

The steep increase in lysosomal activity with the formation of new lysosomes and the clear tendency to accumulate into larger granules (probably autophagic vacuoles), and the late cytoplasmic diffusion of acid phosphatase-positive material, indicate a serious pathological activity of the lysosomes. As they are accompanied by severe changes in the mitochondrial system and in the nuclear elements, it is likely that the tumour cells may be completely destroyed.

Our histological investigations did not clarify the minute development of these changes, but some studies on lysosomal activity in experimental tumours treated with cytoxan and roentgen rays [128–130] have revealed a histological picture of quite the same type—although with less severe changes, and electron-micrographs disclose a pronounced autophagy with destruction of the cytoplasmic elements (mitochondria, Golgi apparatus, etc.) and, in extreme cases, the nucleus.

We find it reasonable to assume that the action observed in our studies is similar to that just described.

Our hypothesis as to the heat effect on the tumour cells differs markedly from the view advanced by Mondovi *et al.* [65] and Giovanella and Heidelberg [66], viz. that the arrest of nucleic acid synthesis is the crucial point. Even if they mentioned that a liberation of lysosomal enzymes may be "capable of carrying out degrading processes in most cellular components and, possibly, in the entire cellular structure", it may be doubted that the lysosomes are the active agent in these processes [69].

Investigations *in vitro/in vivo* have revealed evidence strongly suggesting that the inhibitory effect on tumour growth depends, to a great extent, on the growth rate of the tumour tissue concerned. (Slow-growing tissue needs a considerably higher heat dose than more active tissue [66].) Our histological findings gave no confirmation of this assumption. The heat-treated tumours usually contained a central mass of resting cells, with a small number of mitoses, and cells in active proliferation in the periphery.

In the tumours treated with curative doses, all the malignant cells showed distinct degenerative changes. Individual variations in the severity and speed of the changes were seen in cells of the same type, but there was no marked difference between resting and proliferating cells.

The cellular alterations were of a typical destructive character, with early chromatin condensation and mitochondrial lesions, and the very early lysosomal activity was much more intense than that seen in areas with cellular malnutrition or anoxia.

Only after the application of small heat doses did we observe any difference between central and peripheral tumour tissue. The appearance of cells with "large, dark nuclei" and the activity of acid phosphatase occur mainly in the peripheral parts of the tumour. We explain this as a consequence of the much higher content of acid hydrolases in the active cells in this region than in the central resting cells [131].

The question cannot be solved in the light of our present knowledge.

It must be borne in mind that both the biochemical and the morphological studies are very one-sided; the histological methods are besides very coarse, and a large number of relevant factors are unknown.

A combination of detailed biochemical observations and electron-microscopic and histochemical investigations is highly desirable.

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Isolated Perfused Liver: a Model for Studies on Cancer-Cell Dissemination

A. GUAITANI, J. KVĚTINA and S. GARATTINI
Istituto di Ricerche Farmacologiche "Mario Negri" 62 Via Eritrea, 20157 Milano, Italy

Abstract—*The uptake of cancer cells by isolated perfused liver was studied as a model for cancer-cell dissemination. Ehrlich carcinoma or rhabdomyosarcoma BA 1112 cells were added to the perfusion medium and their disappearance was followed under various conditions. These studies indicated that: (a) cancer cells are rapidly removed by circulation through the liver; (b) there is an increase in vascular resistance which is proportional to the number of cancer cells infused; (c) vasodilatation, induced by nitroglycerine, decreases the uptake of cancer cells by the liver; and (d) an increase of blood glucose and glutamic-oxalacetic transaminase and a decrease in the biliary flow occurs as a result of cancer-cell perfusion.*

THE SPREADING of cancer cells from the primary tumor and the consequent development of metastases represent the essence of the cancer malignancy, hence the need for a better understanding of the complex processes involved in the leakage of cancer cells, their transport in blood or lymph and their uptake by normal tissue.

The lack of suitable methodology to study these problems has been responsible for the slow progress in this important area of cancer research.

Previous work has permitted the establishment of suitable experimental conditions to investigate the adhesiveness of cancer cells [1], the dissemination of cancer cells in the blood [2–6] or in the lymph [7, 8] and the formation of metastases in lymph nodes [9] or in the lung [10].

This paper describes another method of studying the problems related to the passage of cancer cells from the circulation to normal tissue by means of the isolated perfused liver technique [11].

MATERIAL AND METHODS

1. *Animals*

Male rats of Sprague–Dawley or WAG/Rij strains weighing 180 to 200 g, or Albino Swiss male mice, weighing 21 to 24 g were used as donors of blood and liver.

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2. *Cancer cells*

Two types of cancer cells were studied: (a) Ehrlich ascitic carcinoma. The liquid from 7 to 10 days old ascites, transplanted in Swiss female mice, was aspirated into a syringe containing 0·1 ml of 0·2% heparin solution. Cells were sedimented, washed with Krebs-Ringer buffer, suspended in a small volume of the perfusion medium and counted. This cell suspension was added to the medium in the perfusion apparatus, so that the final cell concentration ranged from 2 to 15×10^3 cells per mm^3.

(b) Rhabdomyosarcoma BA1112 cells were grown in Eagle's medium with 10% calf serum for 7 days. The tissue culture was then trypsinized, washed, counted in a Bürker chamber and suspended in the perfusion medium.

3. *Perfusion technique*

The apparatus and details of the technique of the isolated liver perfusion have been described in a previous paper [11]. The technique can be summarized briefly as follows. Homologous heparinized blood of rats or mice was defibrinated and diluted with two volumes of

Krebs-Ringer bicarbonate buffer containing 0·1% of glucose and 3·5% of polyvinylpyrrolidone. The total volume of this perfusion medium was 28 ml for rat and 18 ml for mouse liver experiments. The volume per min passing through the isolated liver was 1 ml/g of liver tissue (about 11 ml for rat liver and about 3 ml for mouse liver). The medium was oxygenated with a mixture of 95% O_2 and 5% CO_2; pH was maintained at 7·4 and the temperature was 37°C. The pulsations of the perfusion pump were 40 to 50 per min giving a basal pressure of 180 to 205 mm of water. Pressure changes in the afferent vessel ("blood pressure") were measured by an electronic manometer incorporated into the apparatus.

4. *Perfusion with cancer cells*

Liver perfusion was usually started with the diluted blood already containing the chosen concentration of cancer cells, but in some experiments the cells were introduced repeatedly into the reservoir of the apparatus. Samples (0·1 ml) of blood, after their passage through the liver, were taken at chosen intervals (1 to 90 min) and diluted with Thürk solution. The cancer cells were then counted with an error lower than 5%. The technique which was used permitted detection of 10 cancer cells per mm^3. No changes in the number of cancer cells were observed when the perfusion medium was circulated for 90 min in the apparatus without the liver.

5. *Biochemical tests*

Hemolysis [12], protein concentration [13]; urea [14], glucose [15], free fatty acids [16], total phospholipids [17], triglycerides [18], cholesterol [19] and glutamate–oxalate transaminase (GOT) [20] were measured in the perfusing blood. Bile volume, bilirubin concentration and BSP test [21] were determined as previously described [11]. Dry weight, proteins, glycogen [22], total phospholipides, triglycerides, cholesterol and succinic dehydrogenase were measured in the liver at the end of the perfusion.

RESULTS

In a series of experiments, Ehrlich carcinoma cells, obtained from mice bearing this tumor in ascites form, were added to the reservoir containing the blood perfusing the isolated liver of rat.

As shown in Table 1, nearly 90% of the cancer cells were trapped by the liver within the first 5 min of perfusion. Since the total volume of the perfusion medium passed through the liver in 3 min, it was concluded, that most of the cancer cells were removed during the first passage through the liver. In the following period (from 5 to 25 min) only a low number of cancer cells, representing less than 1% of the initial concentration remained in the circulation. The high variability found within the different experiments and the rapid rate of disappearance of the cancer cells did not permit calculation of kinetic parameters.

Table 1. Disappearance of Ehrlich carcinoma cells from medium during the perfusion of isolated liver of male Sprague–Dawley rats

Perfusion time (min)	Cancer cells/mm^3 of perfusion medium ± S.E.*	per cent
0	13,900 ± 869	100·0
2	2683 ± 487	19·3
5	916 ± 170	6·6
10	425 ± 89	3·1
15	216 ± 66	1·5
20	113 ± 40	0·8
25	50 ± 22	0·4
30–90	< 10	—

*Mean of 9 experiments.

In order to avoid difficulties arising from the fact that Ehrlich carcinoma cells are xenogeneic to rat liver, it was decided to perform experiments with allogeneic Ehrlich carcinoma cells added to the isolated liver of Albino Swiss mice and with isogeneic rhabdomyosarcoma BA 1112 cells added to the isolated liver of WAG/Rij rats. In all cases 20×10^6 cancer cells/g of liver were added to the isolated livers perfused at a rate of 1 ml/min/g of liver. The results obtained are summarized in Table 2. It is evident that the rate of disappearance of the cancer cells under isogeneic conditions, is slower in respect to xenogeneic or allogeneic conditions, at least in the first 5 min of perfusion.

Microscopical examination of the isolated liver perfused for various intervals with the liquid containing 15,000 cells/mm^3 has shown an acute stasis after 2 min of perfusion with enlarged sinusoids. The neoplastic cells were evident in the portal space. After 30 min, sinusoids were still enlarged and the neoplastic cells were evident in the whole lobular area.

After a liver perfusion of 1 hr the microscopical aspects were normalized, i.e. no stasis could be observed and cancer cells were organized in columns so that they could be distinguished only with difficulty from the normal liver cells.

Table 2. Percentage of disappearance of cancer cells during the perfusion of the isolated liver

Perfusion time (min)	Xenogeneic* condition (%)	Allogeneic† condition (%)	Isogeneic‡ condition (%)
0	100·0	100·0	100·0
2	7·8 ± 3·1	—	29·1 ± 13·1§
5	2·5 ± 0·5	2·8 ± 1·8	9·3 ± 3·7§
10	1·0	2·1 ± 1·6	2·0 ± 1·2
30	0·5 ± 0·3	n.d.	n.d.
60	n.d.	n.d.	—

*Xenogeneic condition (8 experiments) = Ehrlich carcinoma cells and Sprague–Dawley rat liver.

†Allogeneic condition (6 experiments) = Ehrlich carcinoma cells and Albino Swiss mouse liver.

‡Isogeneic condition (5 experiments) = Rhabdomyosarcoma BA 1112 cells and WAG/Rij rat liver.

§ $p < 0{\cdot}05$.

n.d. = cancer cells were below the limit of sensitivity of the method.

Initial concentration of cancer cells: 20×10^6/g of liver.

Diagnosis was possible only because of the irregular nuclei and smaller cytoplasm of the cancer cells.

The presence of cancer cells in the vessels suggested possible changes of blood pressure in the liver. Indeed, a rapid increase of the pressure in the afferent vein was observed during Ehrlich carcinoma cell infusion (Fig. 1) and this rise in pressure was proportional to the initial concentration of circulating cells.

The increase in blood pressure remained high when the total quantity of infused cells reached $55{\cdot}6 \times 10^6$ per 1 g of liver tissue. It should be noted that slow gradual infusion of 40×10^6 cells added in equally divided amounts to the perfusion medium did not induce a comparable increase of "blood pressure" (see Fig. 2).

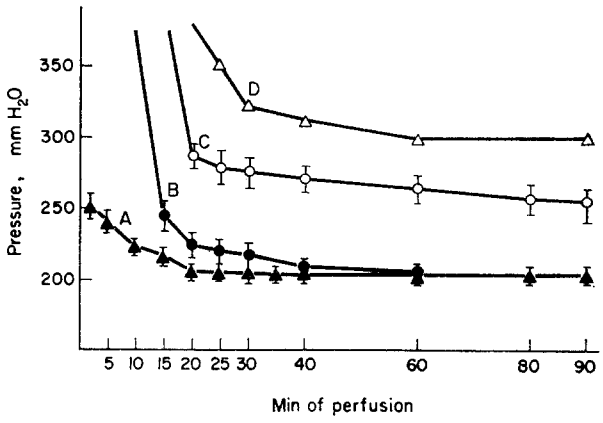

Fig. 1. Blood pressure of rat liver perfused with Ehrlich cancer cells. The vertical bars represent the standard error of the means. The normal pressure before the perfusion of cancer cells was 190 ± 7 *mm* H_2O. *The cancer cells infused per g of liver were* 2×10^6 (*A*), 20×10^6 (*B*), 43×10^6 (*C*) *and* 55×10^6 (*D*).

When the liver was damaged *in vivo* by previous administration of carbon tetrachloride (2·5 ml per kg; 16 and 2 hr before liver isolation) the disappearance of the cancer cells was not influenced. However, histological examination showed large areas of liver necrosis in which the Ehrlich cells appeared less damaged than in control liver.

When the vascular bed was dilated by the addition of nitroglycerine (50 μg per ml of perfusion medium) the rate of disappearance of Ehrlich cancer cells was slower than in controls (see Fig. 3).

This demonstrated the importance of the caliber of the vessels for the passage of cancer cells through the liver. The infusion of pollen grains (Coulter Electronics Ltd) with an average diameter of 13·5 μ, i.e. the same size as cancer cells, caused a mechanical block of the portal space. However, very few particles were found in the sinusoidal liver space and nearly 50% of the particles remained in the

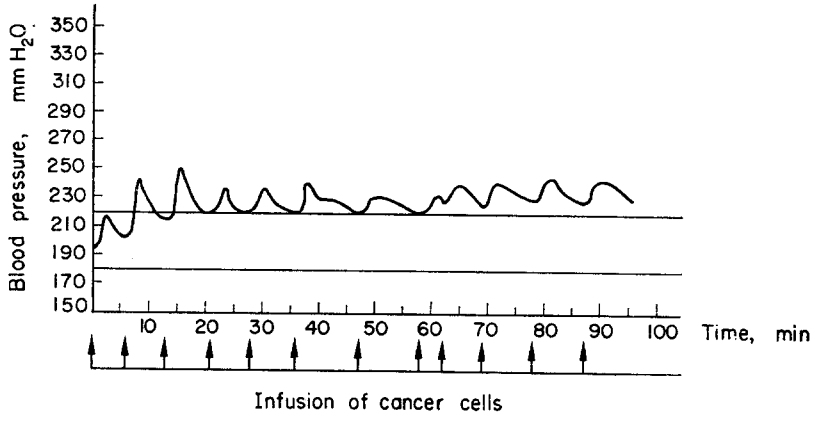

Fig. 2. Blood pressure response to repeated introductions of Ehrlich cancer cells. The total number of cells added was 40×10^6 *per g of liver (in 12 times).*

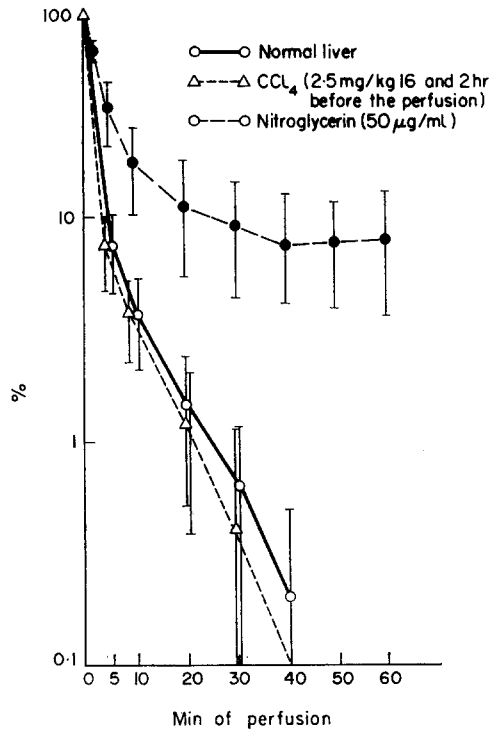

Fig. 3. Clearance of Ehrlich carcinoma cells by rat liver.

circulation for more than 90 min. Other experiments were performed to establish the effect of cancer cells on the function of the perfused liver. For example, high concentrations of Ehrlich cancer cells (55×10^6 cells per g of liver) increased significantly ($p<0{\cdot}01$) the level of glucose and GOT activity in the perfusion medium, (see Table 3).

Bile flow was followed every 30 min during the perfusion. Control bile flow was 5 to 7 μl./min, but with perfusion of cancer cells (43×10^6 cells per 1 g of liver) this flow was reduced by 50%. Cancer cells were not found in the bile, neither were they hydrolyzed when added to bile and incubated for 90 min at 37°C. The BSP clearance [21] was reduced, but BSP concentration increased considerably in the blood when the liver was perfused with cancer cells.

Other biochemical parameters were measured during cell infusion both in perfusion medium (i.e. hemoglobin, urea, proteins, FFA, cholesterol) and in liver tissue (i.e. glycogen, cholesterol, triglycerides, proteins and succinic dehydrogenase) and all of them were found within the limits of normal values.

DISCUSSION

This paper describes the use of the isolated perfused liver as a possible quantitative approach to measure cancer cell dissemination and as a model for chemotherapeutical studies.

The method is based on the determination of the clearance of cancer cells added to the medium perfusing an isolated liver, but it can be applied also to other isolated organs, with particular reference to the lung (unpublished results).

The clearance of cancer cells is very rapid and it is almost complete during the first passage of the perfusing medium through the liver, even when the cell count was 40 million per g of liver.

The immunological situation appears to have certain importance. In fact, by using isogeneic conditions (Rhabdomyosarcoma BA 1112 and WAG/Rij rat liver) the rate of disappearance of cancer cells from the medium was slower than by using an allogeneic (Ehrlich carcinoma and Albino Swiss liver) or a xenogeneic (Ehrlich carcinoma and Sprague–

Table 3. Perfusion of isolated rat liver with various concentrations of Ehrlich cancer cells

Ehrlich cancer cells (number/g liver)	Glucose (mg%) 0 min	60 min	GOT mU/ml 0 min	60 min	BSP clearance* % in blood	% in bile	Bile volume ml/min. 30 min	Liver weight fresh (g)	dry (%)
Control	106	304	22·6	56·7	14·5	14·43	0·159	9·250	28·4
	±3	±13	±1·6	±3·5	±1·7	±2·0	±0·008	±0·540	±0·6
2×10^6	92	343	33·0	62·8	12·0	14·21	0·147	9·150	29·6
	±2	±21	±1·5	±7·9	±2·0	±2·57	±0·011	±0·394	±0·5
20×10^6	88	312	37·1	65·6	11·7	14·9	0·122	9·114	28·5
	±0.1	±6	±5·5†	±7·8	±3·4	±3·42	±0·009†	±0·408	±0·7
43×10^6	89	388	46·0	148·5‡	22·9	6·5	0·082	10·419	27·0
	±0·5	±65	±3·0‡		±3·1	±1·9†	±0·09†	±0·760	±0·6
55×10^6	89	458	51·2	258·9	27·8	0·46	0·030	10·495	26·6
	±0·7	±50†	±5·6‡	±7·3‡	±5·2†	±0·23‡	±0·010‡	±0·154	±0·7

*BSP clearance was measured during the last 30 min of each perfusion. Values of BSP present in blood and in bile are expressed as % of the total amount of BSP added in the perfusion medium (0·2 mg/ml).

†$p < 0{\cdot}05$.

‡$p < 0{\cdot}01$.

Dawley rat liver) conditions. However, no direct conclusions can be made because this difference may simply reflect changes in the capacity of the liver of different species or strains to take up cancer cells. Preliminary histological observations indicate that the cancer cells were trapped by the liver mostly in the sinusoidal and vascular spaces.

Additional studies are required to establish the sequence of events which occur when the cancer cells are cleared from the circulation. Damage to the liver by poisoning with carbon tetrachloride, does not influence the rate of disappearance of cancer cells. However, histological studies indicate that cancer cells present in necrotic areas of the liver appear to be better preserved than those present in normal tissue. The circulation of the liver appears to be an important factor in trapping cancer cells. In fact, vasodilatation obtained by the addition of nitroglycerine to the perfusion mixture caused the cancer cells to remain significantly longer in the perfusion medium.

Cancer cells added to the circulation may considerably affect the blood pressure in the liver.

A direct relationship exists between the number of cancer cells added to the medium and the intensity or the duration of the hypertension. The maximum number of Ehrlich cancer cells which can be infused without changing the blood pressure in the liver is around 1 million cells/g of liver. Higher concentrations, up to 50 million cells/g of liver, result in a hypertension which lasts more than 90 min. However, these figures may change in relation to the speed of perfusion. The higher the hypertension, the more significant is the number of cancer cells passing through the liver and remaining in the circulation. The hypertension seems to be related to a mechanical factor caused by obstruction of the liver capillaries which subsequently increases liver resistance. It is interesting that infusion of pollen grains, of the same size as the cancer cells, results in a very marked hypertension.

It should also be mentioned that the ascitic fluid in which Ehrlich carcinoma cells are suspended does not contain substances showing a vasoconstrictor effect [unpublished data].

Accumulation of cancer cells in the liver results in an alteration of some biochemical parameters in the perfusion medium: there is an increase in the concentration of glucose and transaminase (GOT); bile flow was considerably decreased, although cancer cells were not eliminated through the bile. These effects were present with only 20×10^6 cancer cells per g of liver.

The proposed method for investigating the fate of cancer cells in the liver under controlled conditions has some limitations which are inherent to the technique of liver perfusion.

The arterial circulation (hepatic artery) is not functioning when livers of small animals are used and this may lead to changes in liver tissue which in turn may modify the uptake of cancer cells.

Furthermore, the perfusing medium contains defibrinated blood and therefore it is not possible to evaluate the effect of factors or drugs which may affect dissemination by changing the process of coagulation, considered important according to current knowledge [23–26].

Despite these limitations, the isolated perfused liver can be considered as a model for studying conditions which may enhance or inhibit the passage of cancer cells from the circulation to the hepatic tissue.

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5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC, DIC, NSC-45388)—A New Antitumor Agent with Activity against Malignant Melanoma

STEPHEN K. CARTER and MICHAEL A. FRIEDMAN

Cancer Therapy Evaluation Branch, National Cancer Institute, Bethesda, Maryland

Abstract—5-(3,3-*dimethyl*-1-*triazeno*)-*imidazole*-4-*carboxamide* (*DTIC, DIC, NSC*-45388) *is a new synthetic chemical antitumor agent developed by the Chemotherapy Program of the National Cancer Institute. It was chosen for clinical trial because of activity against mouse leukemia L*1210. *Its mechanism of action is not definitely established but it has been postulated to be both a deranger of* de novo *purine synthesis and an alkylating agent. Clinical trials have shown the drug to be significantly active against malignant melanoma with an overall response rate of* 20·6% (88/426). *This response rate is remarkable for its consistency among various clinical trials. Marginal antitumor activity has been seen against other solid tumors and slightly better activity against sarcomatous disease. Toxicity is mainly to bone marrow and gastrointestinal tract and is reversible in almost all cases.*

INTRODUCTION

THE CHEMOTHERAPY Program of the National Cancer Institute functions in part as a drug development program. One of the most interesting new drugs developed by the program is 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC, DIC, NSC-45388). In 1961 Shealy *et al.* [1] at the Southern Research Institute synthesized 5-diazo-imidazole-4-carboxamide from 5-amino-imidazole-4-carboxamide and reported on its potential antitumor activity. Because of its instability in aqueous solution, disubstituted and monosubstituted triazenoimidazole-4(5)-carboxamide derivatives were synthesized [2–4]. The most active of these triazeno-imidazoles was DTIC [5] and clinical trial was instituted under the sponsorship of the National Cancer Institute. In this paper we would like to review the available data on this compound with emphasis on its clinical activity against malignant melanoma.

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EXPERIMENTAL ANTITUMOR ACTIVITY

In 1962 Shealy [5] reported that DTIC significantly increased the average life span of mice bearing leukemia L1210. Activity against Sarcoma 180, Adenocarcinoma 755 and Ehrlich ascites carcinoma in mice was reported by Hano in 1965 [6]. The activity of DTIC against leukemia L1210 on a variety schedule as reported by Microbiological Associates Inc. [7] working under contract to the Chemotherapy Program is shown in Table 1. These studies do not suggest marked schedule dependency. Alternative treatment schedules were not more effective than daily treatment.

Table 1. *Antileukemic (L1210) activity of NSC-45388 when administered on various schedules*

Treatment schedule*	Optimal dose† (mg/kg)	MST (days)	% increase in survival time over controls
Daily	100	17	89
Every 12 hr	100	15·5	72
Every 2 days	200	17	89
Every 4 days	400	14	55
Every 8 days	400	16·5	84
Day 1 only	400	13	44
Daily for 3 days; respite for 4 days; daily for 3 days, etc.	200	18	100
Untreated controls	—	9	—

*Experiment D-1225: i.p. treatment was started 1 day after i.p. inoculation of 1×10^5 cells in BDF: male mice (25 to 30 g). Treatment was stopped 17 days after inoculation.

†A wide range of logarithmically spaced doses was tested. Only the most therapeutic dose is given.

Data of Hoffman, Kline, Gang, *et al.* Microbiological Associates, Inc.

Wilkoff [8] has suggested a 1° order kinetics model for the killing of L1210 cells by DTIC. That is, the percentage of cells killed at a specific optimal dose is unrelated to the number of cells present. The implication of this is that DTIC appears to be non-cell cycle stage specific, and in this respect DTIC is similar to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and nitrogen mustard and dissimilar to methotrexate, arabinosyl cytosine, 5-fluorouracil and vincristine.

Tryer [9] has compiled data on cross-reactivity of an L1210 strain which is resistant to 5 (or 4)-[3,3-bis(2-chlorethyl)-1-triazeno]imidazole-4 (or 5)-carboxamide (NSC-82196, TIC-mustard, BIC) and found DTIC to be cross-resistant.

Kline [10] has utilized an L1210 clone resistant to DTIC and found sensitivity to TIC-mustard, the nitrosourea drugs, BCNU, Methotrexate, arabinosyl cytosine and cyclophosphamide.

Wodinsky, at Arthur D. Little [Personal Communication], has found that a strain of L1210 resistant to 6-mercaptopurine was still sensitive to DTIC. Kline [11] demonstrated that L1210 mice treated with optimal dosage of 5-fluorouracil and low dose DTIC had a greater survival than either drug alone or a combination of low dose 5-fluorouracil and optimal dose methotrexate.

PHARMACOLOGY

Methodology of assay

Loo [12] has developed a colorimetric determination for the measurement of DTIC in both plasma and urine. This determination is based on the conversion of DTIC to a diazonium compound on exposure to short wave u.v. light. However, Bryan [13] at Wisconsin has modified Loo's method by adding sulfosalicylic acid instead of TCA (as in Loo's original method). Bryan claims his assay provides a narrower range of standard deviations for the samples studied. Saunders [14] has a bioassay model of *B. subtilis* which appears to be satisfactory. Recently, Hunt and Pettillo have reported a microbiologic assay of TC-mustard in an *E. coli* system [15].

Distribution

Loo has done some extensive work elucidating a multicompartment distribution in man and dog systems for DTIC. The drug-distribution fits mathematical models for either a 3-compartment system or a 2-compartment system with an intercompartmental clearance related in a linear way to time [16]. This represents either a 2° or 3° kinetic model. The $T_{1/2}$ for DTIC is about 34 min for an i.v. dosage. Initially, the drug appears to be found in a space greater than the extra cellular fluid, and it can be detected in the CSF in low concentrations (only 14% of serum concentration, which conforms to its relative lipid insolubility).

In pharmacologic doses relatively little drug is protein bound (~5%). Only the i.v. route is practical for administration due to erratic oral absorption (Loo [16]).

Skibba [13] assembled this information concerning administration in man:

Dose (mg/kg)	Method	Serum conc.	Urinary excretion (%)
4·5	i.v. push	8·8±1·8 μg/ml	30
4·5	i.v. infusion over 24 hr	No detectable	30
1·1	i.v. infusion over 8 hr	1·0 μg/ml at 7th hr	30

Both Loo and Skibba found no residual serum levels at 24 hr after administration.

Excretion

DTIC is excreted by a renal tubular secretory mechanism (Loo [16]) rather rapidly. Skibba [17] found that 6 hr after a dose of the drug, 46% of a C^{14} labeled DTIC had been excreted (21% as DTIC and 20% as AIC, 5 amino-4-imidazole carboxamide, a metabolite). Housholder and Loo [18] have clearly shown that the increased amounts of AIC found after an administration of DTIC is formed from the metabolized DTIC.

Metabolism

Loo [19] had identified that one of the highest uptake sites of labeled DTIC is the liver of rats studied. Skibba [17, 20] has implicated N-demethylation in liver microsomal suspensions as a major metabolic pathway, as outlined in Table 2.

This suggests that the microsomal enzyme system of the liver may play a role and inducers of these systems (phenobarbital, dilantin, etc.) might enhance the metabolism of DTIC to AIC.

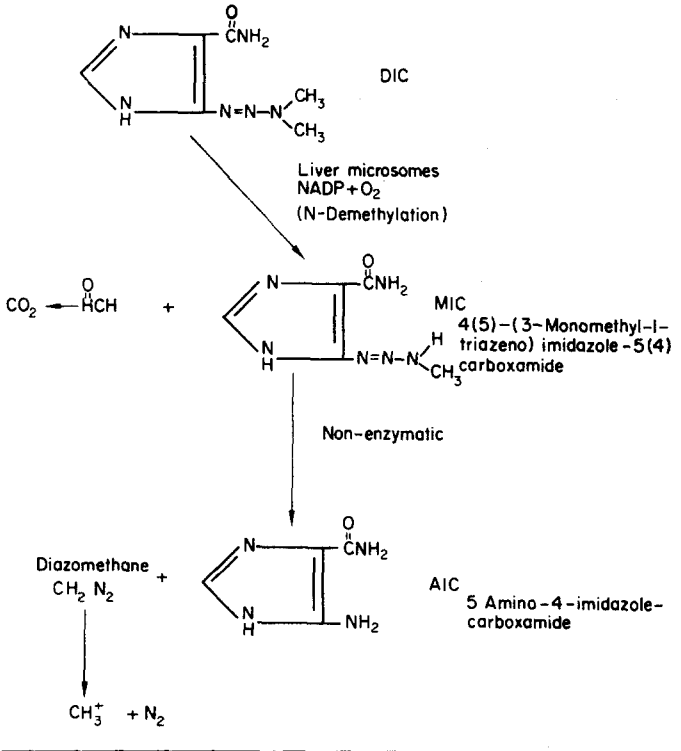
Postulated mechanisms of action

At least three plausible modes of action for DTIC have been proposed.

1. *DNA inhibitor.* Saunders [14] using a *B, subtilis* model studied macromolecules synthesi. and found that while DTIC enhanced DNAs RNA and protein synthesis at low concentrations, at high concentration it inhibited DNA formation. Addition of amino acids also enhanced DTIC activity. Indeed, the imidazole carboxamides, on purely sterio-chemical grounds, would be likely agents for deranging *de novo* purine synthesis.

2. *Alkylator activity.* Skibba [21] suggests that the AIC metabolite is a carrier of diazomethane

Table 2. Suggested metabolic pathway for DTIC



which may act as an alkylator. Hoffman [7] suggests that the increased activity of TIC-mustard over DTIC (both imidazole carboxamide related compounds) may be due to more 'mustard' radical being produced from the TIC-mustard.

3. *SH interaction.* Saunders [14] has also shown that in his bacterial system the DIC inhibition could be reversed by addition of large amounts of glutathione.

Saunders also noted increased activity of DTIC when exposed to u.v. light after it was placed in a bacterial system. Of overall consideration is the postulate of Shealy [4] that imidazole carboxamide is a basic compound which is concentrated to a higher degree in tumor tissue, which is relatively acidic, than in other tissue.

CLINICAL STUDIES

DTIC has been extensively studied against malignant melanoma which appears to be the tumor type most responsive to this drug. A summary of the reported activity to date is given in Table 3. The overall response rate of 20·6% (88/426) is significant in this type of tumor which is notoriously resistant to chemotherapy.

In the data reported by Luce *et al.* from the Southwestern Group [22] there were 21/110 responses (5 complete and 16 partial). Of these objectively responsive patients, 9 had metastatic disease of the liver, 7 to the lungs, 7 to skin, and 9 had localized disease only. The complete remissions were seen in a patient with cerebral and pulmonary metastasis, a patient with pulmonary and lymph node metastasis, in two patients with lymph node disease only and one patient with skin metastases. The median remission duration of those responding in this study were 16+ weeks at last report. Of those who relapsed, the median duration of remission was 17 weeks. Of the patients still alive, survival was 26+ weeks for the responders and 12+ for non-responders. Of those patients expired, survival was 35 weeks for the responders and 12 weeks for the non-responders.

The Eastern Oncology Group study reported by Nathanson [25] comparing DTIC dosage in melanoma is of some interest. In this study, patients were randomized to either a high dose (4·5 mg/kg/day × 10) or a low dose (2·0 mg/kg/day × 10) with a second course given after 20 days of observation. Of 22 evaluable patients given the high dose, 5 obtained PR status, while of 28 on the lower dose, one obtained CR status and 8 PR status. This would indicate that the low dose is as good, if not better, than the high dose and would support the uncontrolled findings of the Central Oncology Group [24] that remissions in melanoma began to be seen at 1·0 mg/kg in their Phase I study. Mean duration of response in the Eastern Group study was 16+

Table 3. Summary of the activity of DTIC against malignant melanoma

Investigator	Schedule used	No. Ev. Pts	No. Obj. Responses	% Responses
Luce, Thurman, Isaacs *et al.* (Southwestern Cancer Chemotherapy Study Group) [22]	250 mg/m²/d × 5 IV repeated *q* 21 days or as soon as tolerated	110	21	19
Central Oncology Group [23, 24]	4·5 mg/kg/d × 10 IV repeated *q* 28 days	160	30	18
Nathanson, Eastern Cooperative Oncology Group [25]	4·5 mg/kg/d × 10 IV	22	5	23
	vs.			
	2·0 mg/kg/d × 10	28	8	28
Skibba, Ramirez, Beal *et al.* [13]	4·5 mg/kg/d × 10	15	5	33
Burke, McCarthy, Milton *et al.* [26]	4·5 mg/kg/d × 10	20	4	20
Accumulated non-published data submitted to Cancer Therapy Evaluation Branch, NCI	4·5 mg/kg/d × 10	71	15	21
	Total	426	88	20·6

weeks for the high dose. Toxity was somewhat reduced in the low dose group with only 35% experiencing nausea and vomiting compared to 75% in the high dose group. Leukopenia was also less at the low dose, but none at the high dose had WBC counts below 1500.

DTIC has been extensively studied in other solid tumors but has shown only marginal activity. The accumulated data reported to the Cancer Therapy Evaluation Branch, NCI, shows responses in 8 out of 81 patients (10%) treated with bronchogenic carcinoma, 8/71 (11%) in patients with colorectal cancer and 4/41 (10%) of patients with breast cancer. Luce [22] has reported responses in 5/32 (22%) with sarcomatous disease including responses in leiomyosarcoma, fibrosarcoma, rhabdomyosarcoma and osteogenic sarcoma. Very preliminary studies indicate significant activity in Hodgkin's disease.

The two frequently encountered organ system sites of DTIC toxicity are the bone marrow and the gastrointestinal tract.

Outlined in Table 4 are the nadirs of leukopenia and thrombopenia observed on the two most commonly used dosage regimens. It is important to clarify that although Luce's experimental design [22] called for repetition of treatment at 21 day intervals, it was often impossible to retreat patients until day 28. Since the average nadir was at day 25, the next course of DTIC was delayed until recovery from leukopenia and thrombopenia was evident. The 10 day treatment schedule of the Eastern Oncology Group evidenced an earlier nadir and recovery time. The tempo of the hematosuppression is fairly predictable. The data from the Central Oncology Group utilizing a daily × 10 schedule also suggests this pattern of hematotoxicity.

Luce also notes that after an initial dose of 250 mg/m^2/day × 5 days, there were 71/91 patients with WBC < 5000, but only 4/91 with WBC < 3000. At this same dosage 8/83 patients had platelet counts < 150,000, while 13/83 < 100,000, and only 5/83 < 50,000. The total clinical experience suggests that it is rarely necessary to transfuse patients with blood fractions, but occasionally the marrow suppression may be severe and lead to fatality. Often an anemia is noted but it is mild and generally insignificant. There have been infrequent reports of a patient experiencing a severe, unexplainable episode of hematosuppression while being treated with an acceptable dosage (an 'idiosyncratic' type reaction).

Table 4. Hematologic toxicity of DTIC

Investigator	Dose	Mean nadir (day)*	Recovery (day)
1. Luce [22] SWCCG	200–500 mg/m^2/day × 5 days *q* 21 days	25	40
2. Nathanson [25] ECOG	4·5 or 2·0 mg/kg/day × 10 days *q* 20 days	7†	20
3. Central Oncology Group [24]	—	—	—

Dose × 10 days	% *Patients* with WBC < 4500
2–3 mg/kg/day	25
3–4 mg/kg/day	39
4–5 mg/kg/day	46

Dose × 10 days	% *Patients* with plateletes < 150,000
2 mg/kg/day	25
3–5 mg/kg/day	25

*From initiation of treatment.
†Occasionally may be delayed to day 20–24.

Symptoms of anorexia, nausea, and vomiting are the most frequently noted of all toxic reactions. Over 90% of patients are affected with the initial few doses. The vomiting lasts 1–12 hr and is incompletely and unpredictably palliated with phenobarbital and/or prochloperazine. Rarely DTIC has caused diarrhea. Some helpful suggestions include restricting the patients PO fluids and food for 4–6 hr prior to treatment. The rapid toleration of these symptoms suggests that a central nervous system mechanism may be involved, and usually these symptoms subside after the first day or two. Rarely intractable nausea and vomiting have necessitated discontinuance of DTIC therapy [22].

There are a number of minor toxicities that are infrequently noted. Luce [22] describes

4/202 patients with a flu-like syndrome of fever to 39°C, myalgias and malaise. This syndrome occurs 7 days after DTIC treatment and lasts 7–21 days, and it may recur with successive treatments. Alopecia was noted in 1/13 patients at the Mayo Clinic [27]. Also, the Mayo Clinic investigators describe facial flushing in 2/13 and facial paraesthesias in 2/13.

There have been no reports of significant liver or renal function test abnormalities.

DISCUSSION

Malignant melanoma is as notorious for its variable response to therapy as it is for its insidiousness, ease and rapidity of metastatic spread and fatal outcome. Local irradiation is only minimally effective and surgical excision is only occasionally successful in producing prolonged survival. Historically, the results of chemotherapy for malignant melanoma have been mostly disappointing. Evaluation of results is made more difficult by the fact that spontaneous regression of individual skin lesions has been known to occur not uncommonly. In a few cases, melanoma is very responsive to drugs, while in many, it is very resistant. As a result, practically every agent used clinically has produced a few responses.

DTIC appears to be the most consistently active chemotherapeutic agent tested against this disease. This is seen when the response rate for DTIC is compared with the pooled data for the commonly used antitumor agents as taken from the English language literature [28]. This is shown in Table 5.

This reveals that DTIC has had the most extensive testing of any chemotherapeutic agent against malignant melanoma and with significantly larger numbers has superior or comparable activity to any other described agent. The responses with DTIC are remarkable in addition for the consistency among various groups studying the drug which is not seen with agents such as hydroxyurea and actinomycin D, despite what appears to be a significant response rate.

Currently combination chemotherapeutic approaches involving DTIC are being evaluated and a new analogue imidazole-4(or 5)-carboxamide, 5(or 4)-[2,2-bis(2-chloroethyl)-1-triazeno] with significantly greater experimental activity [29, 30] is under clinical study.

Table 5. Accumulated data on the commonly used antitumor agents in the single agent therapy of malignant melanoma

Drug	No. Ev. pts treated	No. Obj. responses	% Obj responses
DTIC	426	88	20·6
Nitrogen mustard	34	3	9
Chlorambucil	22	2	9
Cyclophosphamide	37	9	24
Phenylalanine mustard	66	8	12
6-mercaptopurine	30	2	7
5-fluorouracil	42	1	2·5
Methotrexate	27	3	11·1
Arabinosyl cytosine	51	1	2
Vincristine	27	3	12
Vinblastine	54	8	15
Actinomycin D	58	19	33
Mitomycin C	8	1	12·5
Mithramycin	13	2	15
Procarbazine	32	6	18
Hydroxyurea	232	57	24
BCNU	81	15	18·5

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Dose Schedules in Bleomycin Treatment

SVEND JUUL JØRGENSEN
The Fibiger Laboratory, Lundtoftevej* 5–7,
2800 *Kgs. Lyngby, Denmark*

Abstract—*Three different dose schedules have been tested in bleomycin treatment of a methylcholantrene induced dermoid carcinoma transplanted intramuscularly in mice. The reduction of tumour growth was used as a criteria for the therapeutic effect.*

Schedule 1*: Daily injection for* 8 *days.*

Schedule 2*: Injection twice a week for* 3 *weeks.*

Schedule 3*: Cyclic treatment, i.e.* 6 *injections/*48 *hr/week in* 3 *weeks.*

Schedules 1 *and* 2 *gave identical results concerning therapeutic effect and toxicity. Dose schedule* 3 *gave a slight reduction of the therapeutic effect and a marked reduction of the toxicity as compared to schedule* 1 *and* 2*. The result is a better therapeutic index for cyclic treatment.*

IT IS WELL known that the therapeutic index of cancer chemotherapeutic drugs may vary with the dose schedule since the therapeutic effect as well as the frequency of side effects may depend on the schedule used. Goldin *et al.* in 1956 [1] proved that the dose schedule for amethopterin treatment of mouse leukaemia influenced the therapeutic effect and also proved that the optimal dose schedule may be different at different stages of the disease. Since then, several clinical investigations of the importance of dose schedule in the treatment of leukaemias, as well as solid tumours with alkylating agents, folate antagonists, purine and pyrimidine analogs and antibiotics with antineoplastic effect have been reported [2–6]. In radiotherapy too, the time–dose relationships may be important factors [7].

Bleomycin is an antineoplastic antibiotic which in the treatment of dermoid carcinomas has proved more efficient than any drug previously used [8–12]. In the hope not only of improving the therapeutic effect, but also of reducing the frequency of side effects, different dose schedules have been tested in bleomycin treatment of an experimental mouse tumour.

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MATERIAL AND METHODS

Bleomycin† was obtained as bleomycin sulphate in ampoules of 15 mg potency (Lot No. F–18). By biological test, the real amount per ampoule was determined as being equal to 16·3 mg bleomycin A_2. All quantities in the following are referring to one ampoule being 15 mg and not to the real biological amount.

The composition of the lot was: Bleomycin A_1 : 2·7%, B_1 : 3·6%, A_2 : 66·2%, A_2^1 : 1·2%, B_2 : 26·3%, sum of B-group : 29·9%.

The content of one ampoule was dissolved in 15 ml of a 0·9% saline solution, stored at 4°C and used within 48 hr.

The experimental animals were 5 to 9 week-old $STAC3F_1$ mice of both sexes. In the lethality test only male mice were used. The group size is seen in Tables 1 and 2.

The test tumour was passage 41 to 48 of a methylcholantrene-induced dermoid carcinoma. The tumour was derived from a mouse of C3H strain. Originally it was highly differentiated, but after having been grown as a cell culture and retransplanted into mice it shows now no keratinization and it is now a solid carcinoma of a low but stable degree of differentiation.

The tumour was transplanted by the intra-

Table 1

Total dose (mg/kg)	No. of mice	Weight of mice (g ± S.E.M.) Before treatment	Weight of mice (g ± S.E.M.) After treatment	Weight of tumour (g ± S.E.M.)	% Reduction of tumour growth
Dose schedule 1					
41·0	6	19·5 ± 0·4	20·5 ± 0·2	0·52 ± 0·06	64·3
Control	6	19·3 ± 0·4	22·8 ± 0·5	1·42 ± 0·16	
45·4	5	17·6 ± 0·3	19·4 ± 0·4	0·79 ± 0·04	69·3
Control	6	17·3 ± 0·6	21·7 ± 0·7	2·50 ± 0·10	
133·4	6	18·5 ± 0·4	17·3 ± 0·5	0·52 ± 0·09	79·6
Control	6	17·3 ± 0·6	21·7 ± 0·7	2·50 ± 0·10	
172·1	6	17·8 ± 0·5	14·5 ± 0·9	0·22 ± 0·05	87·0
Control	6	19·3 ± 0·5	21·3 ± 0·5	1·45 ± 0·22	
205·1	6	19·5 ± 0·4	13·4 ± 0·7	0·15 ± 0·02	89·3
Control	6	19·3 ± 0·4	22·8 ± 0·5	1·42 ± 0·16	
Dose schedule 2					
46·5	6	25·1 ± 0·3	28·0 ± 0·3	2·27 ± 0·27	42·9
Control	6	26·4 ± 0·4	34·3 ± 0·9	5·62 ± 0·29	
88·9	6	27·6 ± 0·7	27·3 ± 0·8	1·05 ± 0·12	84·6
Control	6	26·4 ± 0·4	34·3 ± 0·9	5·62 ± 0·29	
165·1	6	23·7 ± 0·7	24·2 ± 0·3	1·06 ± 0·16	85·9
Control	6	20·3 ± 0·3	31·9 ± 0·4	7·56 ± 0·22	
226·4	6	22·0 ± 0·6	21·9 ± 0·8	0·79 ± 0·13	89·7
Control	6	20·3 ± 0·3	31·9 ± 0·4	7·56 ± 0·22	
Dose schedule 3					
56·9	6	15·8 ± 0·6	19·8 ± 0·4	3·23 ± 0·33	51·9
Control	6	16·0 ± 0·6	24·9 ± 0·4	6·46 ± 0·23	
109·1	7	16·5 ± 0·3	18·4 ± 0·5	1·33 ± 0·16	72·5
Control	6	16·3 ± 1·1	24·0 ± 0·4	5·16 ± 0·26	
170·6	5	22·8 ± 0·9	22·9 ± 0·4	1·04 ± 0·10	81·1
Control	5	19·4 ± 0·8	31·9 ± 1·2	5·62 ± 0·39	
278·3	6	19·0 ± 0·7	16·5 ± 0·5	0·56 ± 0·06	90·4
Control	6	19·7 ± 0·7	27·1 ± 0·7	5·81 ± 0·22	
361·8	6	19·9 ± 0·8	14·9 ± 0·5	0·32 ± 0·06	94·5
Control	6	19·7 ± 0·7	27·1 ± 0·7	5·81 ± 0·22	
450·0	7	20·0 ± 0·3	15·0 ± 0·6	0·45 ± 0·06	93·3
Control	6	19·6 ± 0·2	29·5 ± 0·2	6·61 ± 0·21	

muscular injection in the right thigh of 0·1 ml of a tumour suspension prepared by mincing the tumour with sharp scissors.

The transplanted tumour was growing logarithmically in the test period (Fig. 1).

In all dose schedules the treatment was started on the day following tumour transplantation. The mice were killed 1 week after cessation of the treatment and the therapeutic efficacy was evaluated by the reduction of tumour growth as compared to the untreated control group. The tumour weight was calculated as the difference between the weight of the right thigh with tumour and the normal left thigh.

The three following dose schedules have been studied using the principles described by Skipper and Schmidt [13].

Table 2. Lethality test

Total Dose (mg/kg)	No. of Mice	Weight of mice before treatment (g ± S.E.M.)	Lethality
Dose schedule 1			
120·0	10	26·1 ± 0·4	0/10
176·8	10	20·6 ± 0·4	2/10
211·2	10	24·7 ± 0·6	7/10
266·4	10	21·0 ± 0·6	9/10
Dose schedule 2			
94·8	10	25·3 ± 0·4	0/10
133·2	10	24·4 ± 0·5	0/10
196·8	10	24·4 ± 0·5	3/10
225·0	10	26·7 ± 0·5	3/10
315·6	9	22·8 ± 0·5	9/9
Dose schedule 3			
210·6	10	25·6 ± 0·4	0/10
270·0	10	25·9 ± 0·4	1/10
365·4	10	24·6 ± 0·6	7/10
475·2	12	22·7 ± 0·3	12/12
541·8	8	23·3 ± 0·4	8/8

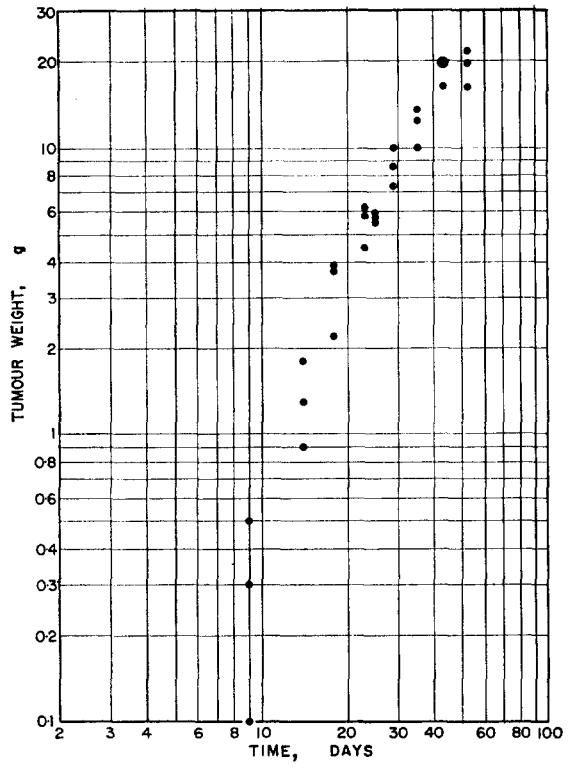

Fig. 1. Growth rate for the intramuscularly transplanted untreated tumour. Each point represents one tumour.

Dose schedule 1: i.p. injection daily for 8 days.
Dose schedule 2: i.p. injection twice a week for 3 weeks.
Dose schedule 3: cyclic treatment with 6 i.p. injections/48 hr/week for 3 weeks.

The LD was studied in all three dose schedules in healthy male mice without tumours. The observation period was 3 weeks after the last injection.

Statistical methods: In all groups, treated as well as untreated, body weights and tumour weights showed curves of normal distribution. The dosage–response curves which were transformed into linear curves, as described by Westerfield [14], were subjected to regression analysis.

The results of this analysis were plotted into probit system together with the results of the lethality tests which were evaluated by probit analysis.

RESULTS

Bleomycin treatment resulted in a significant reduction of tumour growth, using non-toxic doses of the drug according to the three dose schedules described. The figures are shown in Table 1 while the figures for the lethality test are shown in Table 2. After statistical analysis as described above it was found that the dosage–response curves for dose schedules 1 and 2 are identical. The dosage–response curve for dose schedule 3 is found to have a declination different from the declination of the curve for dose schedule 1 and 2 ($p < 0{\cdot}05$).

Concerning lethality, it was found that the results for dose schedules 1 and 2 were identical while the lethality was considerably reduced using dose schedule 3. Curves for dosage–response and lethality are seen in Fig. 2. It appears that at a dose level of 100 mg/kg there was only a minor reduction in the therapeutic effect using dose schedule 3 instead of dose schedule 1 or 2. It is also seen that the difference was diminishing with higher doses.

This means that dose schedule 3, in spite of a slight reduction of the therapeutic effect, offers a better therapeutic index* than dose schedules 1 and 2.

It was found that the therapeutic index for dose schedules 1 and 2 was 0·77 (176/230) while the therapeutic index for dose schedule 3 was 1·04 (280/270).

The same toxic effects as previously reported were seen in this investigation, thus loss of weight, loss of hair and bleeding from foot pads and nails were observed [15–17]. Besides these side effects, toxic liver changes were found. In

*Therapeutic index $= \frac{\text{Maximum tolerated dose (MTD)}}{\text{Minimum effective dose (MED)}}$

as MTD = LD_{10} and MED = T/C 0·10 i.e. the dose inhibiting tumour growth to 10% of the untreated controls [21].

almost all survivors in the lethality test the liver showed signs of degeneration at autopsy after the observation period. In early stages the liver was found to be enlarged and yellow. In later stages it was found to be small, atrophic and lobulated. Microscopically there was found an acute toxic parenchymatous degeneration.

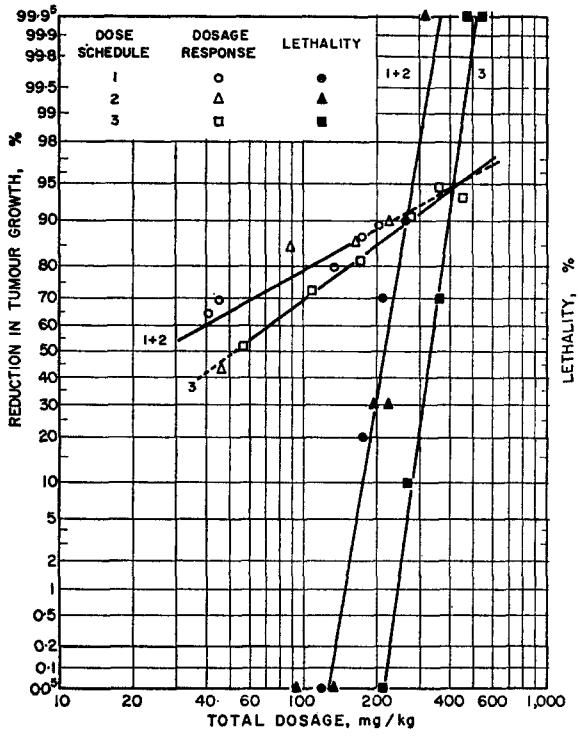

Fig. 2. Dosage–response curves and lethality curves in probit system. The curves for dose schedule 1 *and* 2 *are found to be identical and significantly different from the dosage-response and lethality curves for dose schedule* 3. (*t-test on difference between inclination of dosage-response curves:* $p < 0{\cdot}05$, *t-test on difference between lethality curves:* $p < 0{\cdot}01$).

The liver degeneration was in some cases accompanied by haemorrhagic ascites. In some cases fibrinous peritonitis was seen, probably the result of a local irritative reaction.

Special interest was taken in the lungs as previous investigations of the distribution of bleomycin in the organism have proved that the drug is concentrated there [16]. Also several clinical observations of lung complications have been reported [11, 12, 18–20]. However no toxic lung changes were observed in the present investigation.

DISCUSSION

Investigations of the mode and time of action of cytostatic compounds related to cell cycle are fundamental in establishing an optimum dose schedule.

It has been found that the sensitivity to bleomycin of the proliferating cell is greatest in late G_1 and early S-phase, i.e. the time of DNA synthesis [21, 22], and also that bleomycin causes breaks of DNA *in vitro* and *in vivo* [23–26]. Consequently, there may be two effects of bleomycin in tumour cells, partly an inhibition of DNA synthesis in proliferating cells and partly an injury of DNA in cells not in DNA synthesis. No statmokinetic effect of bleomycin has been reported.

It has been found that bleomycin is accumulated in the organism when given daily. As injection every third day did not cause accumulation [17], it has been recommended to give bleomycin twice a week as therapy for humans. However, it is conceivable that further reduction of toxicity without reducing the therapeutic effect might be obtained by a cyclic dose schedule. In the present work 6 injections were given in 48 hr according to dose schedule 3. During this time bleomycin will accumulate in the organism and in the tumour. If the individual doses are sufficiently high, a cytostatic concentration might thus be obtained lasting until the next cycle. However, the steeper course of the dosage–response curve for dose schedule 3, as compared to schedules 1 and 2, indicates that this level is not reached at the lower doses studied in the present work. On the other hand the free intervals between the cycles are able to reduce toxicity and cause a better therapeutic index for the cyclic treatment.

There might be some reduction of the number of cells entering into late G_1 and S-phase during the series of injections because of the ability of bleomycin to cause scission of DNA in the cells not in DNA synthesis. Up to now the effect of bleomycin on the size of growth fraction and other cytokinetic parameters is unknown.

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The Effects of Divalent Cations on the Ionic Permeability of Cell Membranes in Normal and Tumour Tissues

I. G. F. GILBERT

Department of Physics, Institute of Cancer Research (Surrey Branch), Belmont, Sutton, Surrey, England

Abstract—*It is shown that the ability of three strains of transplanted rat tumours to maintain cellular potassium contents on incubation in media devoid of calcium salts is due to a tumour calcium requirement which is satisfied by concentrations of at least an order of magnitude less than required by the normal tissues yet studied. The evidence is that potassium loss by the tumours on incubation in the presence of very low free calcium ion concentrations maintained by metal-ion buffers, is due to an increase of plasma membrane permeability, similar to those which are believed to occur in normal tissues where the calcium ion "thresholds" are much higher. The cell membranes of the tumours are thus more resistant to lowering of the calcium ion concentration than the normal tissues. Differences also occur in the extent to which other divalent cations, in particular magnesium, can substitute for calcium.*

IT HAS been reported that two strains of transplanted animal tumours can maintain their intracellular potassium concentrations unchanged on incubation in media devoid of calcium or magnesium salts (Ehrlich mouse ascites cells [1] and Morris hepatoma 5123 [2].) This behaviour contrasts with the generally accepted pattern for normal tissues. Thus, for the considerable number of normal tissues studied, it has been found that cellular potassium is readily lost if the extracellular calcium ion concentration falls below 1 to 2 mM (including, of mammalian tissues: brain cortex [3], kidney cortex [4], liver [2, 5], smooth muscle [6], diaphragm [7], erythrocytes [8, 9] and cardiac muscle [10]. Higher than normal calcium concentrations can also improve potassium retention in less favourable conditions of incubation [11]. It is generally thought that the function of calcium in preventing cellular loss of potassium, and possibly of other small molecules [12], is to reduce the ion-selective permeability of the plasma membranes [13]. It is not clear from the results reported on the tumour tissues whether the absence of a calcium requirement in these tumours reflects a fundamental difference of cell membrane permeability properties or whether there is a trivial explanation (e.g. a relatively high tumour calcium content, as suggested by Kalant and Hickie [2]). The present work aims at distinguishing between these possibilities. Three tumour systems have been studied, chosen for their different tissue origins, bone, kidney and breast, and widely different states of transplantation, from a first transplant to a series in over the 250th transplant.

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EXPERIMENTAL

The incubation and other techniques have have been described in detail [14]. Slices of rat tissues, 0·3 mm thick, were incubated at 38°C in conditions which previous work on liver and tumour slices had established as giving tissue potassium and water contents, in steady state conditions, within the normal *in vivo* ranges. The inorganic composition of the complete medium was close to that of Krebs–Ringer–phosphate–bicarbonate, with additions of glucose and a colloidal material (inulin). Actual concentrations, as made up, were:

Na, 147; K, 3·7; Ca, 2·46; Mg, 1·1; Cl^-, 119; HCO_3^-, 23·8; $SO_4^=$, 1·1; $H_2PO_4^-$, 1·8; HPO_4^-, 7·3; glucose, 5·2; (all the preceding in mM); inulin, 20 g/l. The complete medium contained both Mg^{++} and Ca^{++} at concentrations close to those in mammalian extracellular fluid. In the first experiments only Ca^{++} was changed in concentration or replaced by a different divalent cation. The presence of Mg^{++} was found to affect some of the results and two series of normal – Ca^{++} control media were therefore used for liver incubations, firstly with Mg^{++} present in normal physiological concentration and secondly without Mg^{++}. For the tumour systems incubation media containing only one divalent cation, or none, were used, since the high response to Mg^{++} prevented comparison with other cations in its presence. Tumour slices were incubated for 2½ hr, which was found sufficient for the potassium distribution to reach a steady-state. Liver slices, which undergo a greater initial loss of K, were incubated for 4½ hr for steady-state conditions to be established [14]. In other experiments, in identical conditions, liver slices were able to maintain their K content after re-accumulation, and also their Rb content, which is a more sensitive index of energy metabolism [14], to as long as 7½ hr (graphs of the time course of potassium content are given in [14]). Very rapid stirring of the slice suspensions was found essential, and was provided by a stream of humidified 95% O_2–5% CO_2 from the bases of the incubation tubes. The ratio of medium volume to tissue weight was kept high to minimize the effects of the tissue on medium composition, and to provide an ample supply of divalent cations. This ratio was 100–200 to 1, and in normal incubation conditions the tissue calcium represents about 2% of the total in the medium. The divalent cations and complexing agents were added before the start of incubation. At the end of incubation, slices were rapidly drained on coarse, sintered glass discs, were weighed before and after drying, and were dissolved in concentrated HNO_3, preparatory to K analysis. K and Ca analysis were carried out by flame photometry.

There are two sources of Ca^{++} contamination of the incubation media: firstly, the impurity content of the salts used (principally of the sodium chloride), and secondly the incubated tissue itself. The first source is expected to contribute about 0·01 mM of calcium, using the manufacturer's specification. An upper estimate of the second contribution may be made if it is assumed that those cells which are sectioned during preparation of the slices lose all their calcium to the incubation medium. An estimate for liver tissue from cell and slice dimensions (taking the cell diameter as around 20 μ) is that about 10% of cells are so sectioned and using the tissue concentration of 4·1 ± 0·2 mM/kg wet weight, and the ratio of tissue weight to the volume of medium, this gives an upper limit again of about 0·01 mM of Ca contaminant. Since this second contribution may well be overestimated it is likely that the total Ca contamination in nominally Ca^{++}-free media is of the order of magnitude of 0·01 mM or a little greater. More precise control of such low medium Ca^{++} concentrations is possible by using complexing agents as metal-ion buffers and this approach is used in the present experiment. The essence of this procedure is that a "pool" of total medium calcium is present in sufficient excess to swamp those binding sites of particular interest, in this case the cellular sites (as calculated above), and the free Ca^{++} concentration is controlled by the complexing anion, which must be present in sufficient excess of the total calcium to provide adequate buffering power.

The tumours were:

BICR/A15, originally a DMBA-induced kidney tumour in "August" strain rats. This was a very anaplastic tumour, and was studied in its first transplant.

BICR/A3, originally a radiation-induced osteosarcoma in "August" rats, studied in its 26th and 27th transplants.

BICR/M1, in "marshall' strain rats [14, 15], which originated as a spontaneous mammary fibrosarcoma, and had undergone over 250 transplants (both the A15 and A3 tumours had been preserved for a period at 77°K).

RESULTS

A normal tissue: slices of normal liver

Liver was selected as an example of normal tissue for more detailed study, as in previous tissue comparative studies of other aspects of alkali metal metabolism [14], and in view of the considerable amount of experimental work which has been carried out on ion transport in this tissue.

The effects of varying concentrations of medium calcium, the correlation of medium calcium and slice calcium concentrations, and the effects of other divalent cations in mimicking the action of Ca^{++} were investigated. The results are given in Figs. 1 and 2 and in Table 1. The steady-state concentration of potassium

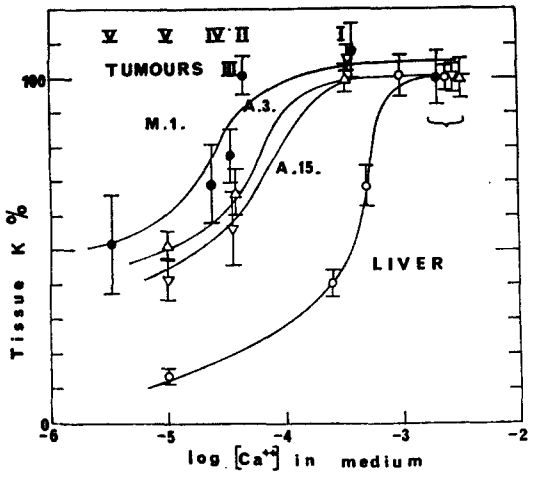

Fig. 1. *Steady-state potassium concentration (% of controls with normal extracellular Ca^{++}) in slices of three transplanted rat tumours and in normal liver, plotted against the free Ca^{++} concentrations of the incubation media. Compare with Table* 1 *for actual tissue K concentrations. The Ca^{++} concentrations of the liver incubation media represent the amount of Ca salt added: the lowest Ca^{++} concentration has been estimated as described in the text for the M.*1. *tumour. The Ca^{++} concentrations in the tumour incubation have been reduced by addition to normal Ca media, in order, of: citrate (I) NTA (II); citrate+NTA (III); and in nominally Ca-free media by addition of succinate (IV) and citrate (V). The actual free Ca^{++} concentrations have been calculated from equilibrium constants as described in the text, the lowest Ca^{++} concentration in each case in a similar way, using the estimated level of Ca contamination (in the text).*

*M*1 *Tumour,* ●; *A*3 *Tumour* △;

*A*15 *Tumour,* ▽; *Normal liver,* ○.

in the slices is dependent on the Ca^{++} concentration in the medium and is reduced by about 50% when the Ca^{++} concentration is reduced from the normal control level of 2·46 mM to about 0·36 mM. At this, and at much lower medium calcium concentrations, the slice calcium content remains within the normal *in vivo* range (Fig. 2). The results of Table 1 show that K^+ retention is also supported by other divalent cations, the order of effectiveness in the presence of one divalent cation being

$$Ca^{++} > Sr^{++} > Co(NH_3)_5Cl^{++} > Mg^{++} > 0.$$

Large organic cations, for example 2:7 diamino fluorene and rosaniline (3+), also

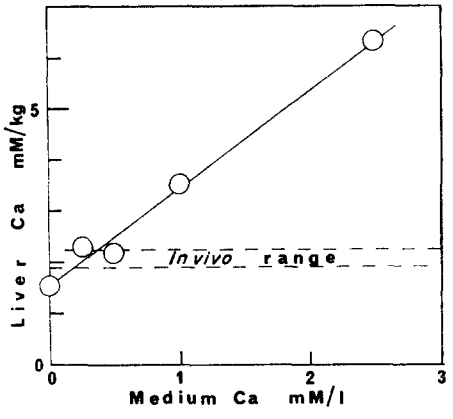

Fig. 2. *The dependence of the calcium content of rat liver slices on the calcium concentration of the incubation medium. Each point represents one bulked tissue sample.*

Table 1. K *concentrations (mM/kg wet weight) in incubated slices of* 3 *transplanted tumours and normal liver in the presence of various divalent cations and complexing anions*

Added divalent cation (2·5 mM)	Basic medium +1·1 mM Mg^{++}	Basic medium	Basic medium	Basic medium +10 mM succinate	Basic medium +1·5 mM EDTA	Basic medium +5 mM citrate		
	Liver		M1 Rat tumour				A3 Tumour	A15 Tumour
Ca^{++}	87±13 (40)	96±2 (13)	83±6 (4)	100±9 (8)	93±7 (8)	109±9 (12)	103±4 (12)	83±3 (6)
Mg^{++}	54±4 (8)	49±3 (16)	90±19 (10)	105±4 (8)	89±9 (8)	109±9 (17)	97±8 (6)	84±7 (4)
Sr^{++}	89±9 (8)	78±2 (6)	84±9 (4)	—	—	97±12 (14)	96±6 (6)	63±2 (6)
$Co(NH_3)_5$ Cl^{++}	64±5 (8)	57±5 (13)	72±8 (4)	89±2 (6)	42±2 (4)	72±6 (12)	77±6 (12)	30±7 (6)
Ca^{++} +NTA (5 mM)	—	—	—	—	—	78±9 (12)	68±7 (6)	44±9 (5)
None	30±8 (60)	35±4 (11)	72±10 (10)	69±12 (4)	47±6 (4)	52±15 (17)	53±4 (10)	32±5 (6)
Rosaniline^{3+} (1 mM)	67±2 (4)	—	—	—	—	46±7 (6)	—	—
2 : 7 diamino-fluorene (1 mM)	71±1 (4)	34±3 (12)	—	—	—	—	59±6 (6)	—

Standard deviations are given, with the number of samples of bulked slices in brackets. The "basic medium" contained no added divalent cations; addition of 1·1 m Mg^{++} and 2·5 m Ca^{++} brings its composition to that given in the experimental section. Control *in vivo* K contents were: M1 Tumour, 82±3; A3 Tumour, 103±6; A15 Tumour, 96±4: Liver, 89±1.

had considerable effectiveness in supporting potassium retention (Table 1), in the case of diamino-fluorene only in the presence of the minimally-active Mg^{++}.

The M1 tumour

In contrast to the results with liver slices, omission of calcium salts from the incubation medium had no effect, and the omission of both calcium and magnesium salts caused only a small fall from 83 ± 6 to 72 ± 10 mM potassium per kg wet weight of slices of M1 rat tumours in steady state conditions (Table 1). It seemed likely that K retention in nominally Ca^{++}-free conditions was due to the presence of low concentrations of Ca^{++} contaminant, as estimated in the experimental section. To overcome this difficulty, the free divalent cation contents of the incubation media were controlled by metal-ion buffers as described in the experimental section. In Fig. 1 and Table 1, results are given in which the free Ca^{++} concentration is controlled by succinate, citrate, nitrilotriacetate (NTA) and ethylenediamine-tetra-acetate (EDTA) (equilibria have been calculated from appropriate data in [16]). From Fig. 1 it is seen that no fall in potassium retention occurred unless the free Ca^{++} concentration was reduced to 0·035 mM or less. The fall in potassium content in nominally Ca- and Mg-free media, but in the absence of complexing anions (Table 1), corresponds to a free Ca^{++} concentration of about 0·03 mM (Fig. 1), which accords with the order of magnitude estimate of Ca contamination given above. Since the incubation conditions were similar in all experiments this value has been used to calculate Ca^{++} concentrations in nominally "Ca-free" media in the presence of the complexing agents (Fig. 1). Direct inhibitory effects by the complexing agents themselves are ruled out for reasons given in the discussion.

Since citrate is a normal intracellular constituent and appears to have no inhibitory effects in the experimental conditions, citrate-containing media were used to reduce the effects of contaminating divalent cations, thus permitting comparison of the relative effects of different cations with a higher level of statistical significance than otherwise possible (Table 1). (There is the additional advantage in using this complexing agent that its association constants with Ca^{++} and Mg^{++} are very nearly equal [16]). In these conditions the equal effectiveness of the cobaltammine and rosaniline cations are shown more clearly (Table 1).

The A3 and A15 rat tumours

Similar experiments were carried out with slices of these two tumours. The results were quantitatively similar to those with M1 tumours (Fig. 1 and Table 1): reduction of the free Ca^{++} concentration in the incubation medium to 0·3 mM, by addition of excess citrate, caused no loss of potassium (0% and an insignificant rise of 5% respectively): potassium loss was apparent only at medium Ca^{++} concentrations of 0·04 mM or less; Mg^{++} and Ca^{++} were nearly equally effective in preventing potassium losses: and the cobaltammine and, in the A3 tumour, 2 : 7 diamino fluorene also, had little if any, effect.

The effect of low Ca^{++} conditions on the water contents of slices of the tumours and of normal liver

The results of Figs. 1, 2 and Table 1 are given in terms of tissue wet weight, since this provides a more stringent test of experimental technique than dry weight values. The dry weights of tissue samples were also measured routinely and values are given in Table 2, in a form to allow comparison with the appropriate columns of Table 1. For the A3 and A15 tumours and for normal liver it will be seen that maximal K losses occur without significant change in the dry matter content of the slices. In the M1 tumour considerable K loss occurs with no change in the dry matter content (the Ca^{++} + NTA and cobaltammine results) but at the lowest Ca^{++} concentrations a slight drop is apparent. Changes in liver dry weight are apparent in the presence of the organic cation 2 : 7 diamino-fluorene and the chloro pentammino cobaltic ion.

DISCUSSION

The present results show that cells of three lines of transplanted tumours, of quite different tissues of origin (kidney, bone, breast) and states of transplantation, are still able to maintain their normal intracellular potassium concentrations when the calcium ion content of the incubation medium has been reduced to only trace concentrations. The observations of Levinson [1] and Kalant and Hickie [2] provide two other examples of the same phenomenon associated with tumour cells (Ehrlich ascites carcinoma and Morris hepatoma 5123). In contrast as shown in Fig. 1, preparations of normal liver require exogenous Ca^{++} at a concentration twenty times higher than that required by the tumour preparations. Published data for a variety of other normal tissues (listed in the introduction) show that the calcium ion

Table 2. Dry matter contents (% of wet weight) in incubated slices of 3 transplanted tumours and normal liver, in the presence of various divalent cations

Added divalent cation (2·5 mM)	Basic medium	Basic medium + 5 mM citrate		
	Liver	M1 tumour	A3 tumour	A15 tumour
Ca^{++}	29·8 ± 0·7	17·6 ± 0·8	18·6 ± 0·6	19·8 ± 1·2
Mg^{++}	29·2 ± 0·7	17·7 ± 1·1	18·8 ± 0·7	20·2 ± 1·7
Sr^{++}	30·4 ± 0·6	17·6 ± 1·2	18·9 ± 0·6	18·9 ± 1·3
$Co(NH_3)_5 Cl^+$	*24·2 ± 0·9	16·9 ± 1·8	18·2 ± 0·4	20·9 ± 2·4
Ca^{++} NTA	—	17·3 ± 1·2	18·4 ± 1·0	19·0 ± 0·6
None	30·4 ± 0·6	*16·2 ± 1·6	18·2 ± 0·6	18·8 ± 1·5
Rosaniline^{3+} (1 mM)	—	*16·1 ± 0·6	—	—
2 : 7 diaminofluorene (1 mM)	*27·6 ± 1·5	—	19·9 ± 0·4	—

*Significantly reduced below Ca^{++} controls.
Standard deviations are given. The potassium contents and numbers of samples are given in Table 1.

requirements are of similar magnitude to those found here for liver [2–11, 13]. These findings represent a substantial qualification, as regards tumour tissues, of the conclusion that "extracellular calcium ion is of universal importance in maintaining low intracellular sodium and high intracellular potassium in living systems" [18].

As listed above the mammalian tissue systems showing a low Ca^{++}-requirement have in common that they are all transplanted tumours. In particular the hepatoma studied by Kalant and Hickie was a carcinoma of hepatic parenchymal origin [2] and it would seem therefore that the reduced Ca^{++}-dependence originated at some stage in the development of the tumour (cf with normal liver results in Fig. 1 and [2]). A low Ca^{++} requirement is shown by a tumour in its first transplant (the A15 tumour), which suggests that repeated transplantation is not a necessary prerequisite. The low Ca^{++}-requirement of this kidney tumour can also be specifically contrasted with that of its tissue of origin [4], although the cellular origin of the tumour is uncertain.

A further finding of the present experiments is that the greater effect of low concentrations of Ca^{++} in maintaining intracellular K^+ in the tumour systems is associated with different relative effectiveness of other divalent cations. In particular in all the three tumours studied Mg^{++} is as effective as Ca^{++} at concentrations of 0·35 mM (Table 1 and Fig. 1). In this respect the Morris 5123 hepatoma also shows an equal effect of Mg^{++} and Ca^{++} at the concentrations studied by Kalant and Hickie [2], in contrast to normal liver (Table 1 and [2]), where the effect is more nearly Ca^{++} specific. A similar Ca^{++}-specificity has also been reported for erythrocytes [17] and is implied by results for slices of normal rat kidney cortex [4]. There is however little information for other normal tissues and the base of comparison of these tumours with normal tissues in respect of the Mg^{++} effect is therefore narrower than for the reduced Ca^{++}-dependence.

There are two types of possible mechanism by which low-Ca^{++} conditions could lead to potassium loss from tissue preparations: partial inhibition of transport enzymes or by an increase in the permeability of plasma membranes to alkali cations. For a number of cells and tissues, electrophysiological investigations have provided direct evidence of such an increase in cation permeability of cell membranes in media of low Ca^{++} concentration [19, 20]. These tissues include mammalian smooth muscle [21, 22] and kidney cortex [4], which retain lowered contents of potassium in such conditions [6, 4]. For frog muscle [23, 24] the electrical experiments were followed-up by determinations of radioisotope fluxes, and an increased rate of efflux of ^{42}K (by a factor of about two) was shown directly. The present findings that dry matter contents of the tissues were unchanged in conditions of considerable potassium loss (Table 2) implies that the permeability to other solutes (loss of which would affect intracellular osmotic pressures) was little affected.

There are some indications that if an enzymic effect contributes to the experimental findings (that is by a partial metabolic inhibition at low extracellular Ca^{++} concentrations) it is not a direct one. Intracellular inhibitory effects seem ruled out, since free Ca^{++} concentrations in cells are considered to be very low [28], and below the minimum extracellular values plotted in Fig. 1. In liver also, tissue calcium contents are within the normal range at extracellular Ca^{++} concentrations which result in considerable K loss (Fig. 2). Further,

calcium is inhibitory and not stimulatory to both glycolytic [25] and respiratory enzymes [26], and can show inhibitory effects towards cell membrane ATP-ase at concentrations as low as 0·2 mM [27]. The equality of the effects of magnesium and calcium ions in the tumours (Table 1) also argues against an enzymic action, since these ions usually have quantitatively different effects in enzyme activation. In liver the (partial) activity of the cobaltammine and organic polyamino compounds in replacing Ca^{++}, almost entirely rules out an enzymic site of action. A further way in which enzyme inhibition may be brought about in the tumour preparations, is by the action of the complexing anions. However, the smooth variation of the tumour potassium contents with the free calcium-ion concentrations, in the presence of a variety of complexing anions (Fig. 1), implies that the major variable is the free calcium ion concentration and not the presence of complexing anion. Moreover, citrate, when present in excess, has no effect on the potassium content unless the total calcium concentration is also greatly reduced (Table 1). It is therefore likely that a major part of the K^+ losses shown in the present experiments is due to increases in the permeability of the plasma membranes to alkali cations. There are however two qualifications: (1) the present experiments (and other available data) do not provide direct evidence for such a permeability increase in the particular tissues studied here; and (2) it is not certain that "passive" ionic permeability and active transport are completely separable (the theory used by the present author in interpreting steady-state potassium and rubidium distributions and isotope fluxes illustrates this point [14].

The nature of the interactions between calcium ions and plasma membranes which affect the ionic distributions is not established. Structural changes have been shown by electron microscopy to occur throughout the plasma membranes of liver cells in the presence of complexing agents, including citrate [29], but the relationship of these changes to those in ion transport and permeability is not clear. It is reasonable that calcium ions exert their action on cell membranes at "receptor-sites", but the molecular nature of these is not known: cases can be made out for protein [30], polysaccharide [31] and phospholipid [32] calcium-receptor sites.

The results do not give information about the relative numbers of Ca^{++}-receptor sites or the amount of calcium bound, but are perhaps best interpreted in terms of a higher apparent affinity of the sites for Ca^{++} in the tumour system. An increased density of surface negative change could increase the apparent affinity, but would not be expected to alter the relative affinities for, for example, Ca^{++} and Mg^{++}. It is therefore possible that the receptor sites may be physico-chemically different in the tumours, which may be open to investigation by chemical analysis. An alternative possibility is that the receptor sites are an intrinsic part of cell membrane structure as in phospholipid monolayers where cation-binding specificity can depend on the packing density of the anionic groups [33].

The functional significance of the present results and their relationship to the development of malignancy are problems which are raised, rather than answered, by the present paper, and their solution may require a much greater understanding of cell membranes than presently exists. The experimental technique, using tissue slices, is also not well suited to studying these problems in depth, as exemplified above in the case of the A15 kidney tumour. The findings do suggest however that the divalent cation requirements of functioning cell membranes may provide, at least, a method of probing membrane changes during tumorigenesis.

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Isolation in Newborn Mice of Tumour Cell Variants from a Polyoma-Transformed Non-Malignant Cell Line

E. HUNTER and G. NEGRONI

Department of Environmental Carcinogenesis, Imperial Cancer Research Fund, Burtonhole Lane, London, N.W.7

Abstract—*Tumours developed in a proportion of newborn C3H/Bi and F1 C3H/Bi x C57BL hybrid mice inoculated with a polyoma virus-induced tumour cell line, non-tumourigenic in adult syngeneic mice.*

Some of the tumours were transplantable into adult mice, others were not. Tissue culture cell lines isolated from the transplantable and non-transplantable tumours differed in their tumourigenicity for adult mice and in their immunological properties. The relevance of these findings to tumour progression is discussed.

INTRODUCTION

INOCULATION of polyoma virus (Py) into a newborn C3H/Bi mouse gave rise to a transplantable subcutaneous sarcoma. Single cell cloning of this tumour in tissue culture allowed the separation of two lines of cells: one, the transformed malignant line, produced tumours in adult syngeneic mice, while the other, the transformed non-malignant line, did not [1]. The serum of adults inoculated with the non-malignant cells contained cytotoxic antibodies to a high titre [2] as demonstrated in the *in vitro* colony-inhibition test of Hellström and Sjögren [3]. Some cells, resistant *in vitro* to the action of this specific antiserum, gave rise to progeny that remained insensitive through serial transfers in tissue culture, and were now malignant in adult mice [2].

Thus, susceptibility to cytotoxic antiserum may be lost, or greatly reduced, in a small minority of a cell population maintained *in vitro*. This paper shows that similar changes may occur *in vivo* when these transformed non-malignant cells are inoculated into newborn, immunologically incompetent mice.

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MATERIAL AND METHODS

Transplantation

For subcutaneous and intraperitoneal inoculations pure line C3H/Bi and F1 hybrid C3H/Bi x C57BL mice were used either as adult or newborn.

Tumours were transplanted by inoculating suspensions of tissue in Hanks' balanced salt solution, 1 : 5 v/v. Positive mice were killed within 2 weeks: negative mice were kept under observation for 1 year.

Tumourigenic activity was measured by inoculating groups of 10 mice, each group with a serial 10-fold dilution of cells, (10^6–10^2). The 50% tumour production was calculated by the Reed–Muench method.

Cells

The isolation of the transformed non-malignant cell line (7_2) was described previously by Walls and Negroni [1]. When these cells were inoculated into newborn mice tumours appeared from which we isolated four sublines in tissue culture; complete immunological studies were carried out on only two:

(a) Fibrosarcoma—non-malignant subline (E38);
(b) Fibrosarcoma—malignant subline (E20).

Tissue cultures

All cell lines were grown in Esco plastic petri dishes in Eagle's Medium (MEM) containing 10% calf serum and antibiotics (100 i.u. penicillin and 100 μg streptomycin/ml).

The lines were subcultured twice weekly after treatment with 0·25% trypsin in phosphate-buffered saline (PBS), pH 7·2 at 37°C for 5 min; single-cell suspensions were counted in a haemocytometer and seeded at concentrations of $2–3 \times 10^5$/10 ml of MEM in 100 mm Esco plastic petri dishes and 1×10^5/4 ml of MEM in 60 mm Esco plastic petri dishes. Cells were incubated at 37°C in an atmosphere of 5% CO_2.

Immunological reagents

The antiserum against the non-malignant line (7_2) was prepared in C3H/Bi mice by intraperitoneal inoculation of 1×10^6 cells, twice weekly, for 3 weeks. The mice were anaesthetized, and bled from the subclavian veins; the serum was separated by centrifugation. The serum reacted specifically with cells from Py-induced mouse tumours and had a 50% inhibition titre of between 1 : 64 and 1 : 128, when tested in the colony inhibition test with the non-malignant cell line (7_2).

Control sera were obtained from untreated C3H/Bi mice.

The sera contained no haemagglutination inhibiting antibodies for polyoma virus.

Fresh guinea pig serum stored at −20°C was used as complement.

The colony inhibition test

To compare the colony inhibiting properties of immune and normal serum, 0·1 ml of serum diluted with an equal volume of PBS (pH 7·2) was mixed with 0·2 ml of MEM containing the specified concentration of cells. The mixtures were incubated in a water bath at 37°C for 1 hr, shaking frequently and then 0·6 ml of fresh guinea pig serum was added. The preparation was shaken at intervals during a further period of 45 min at 37°C.

To estimate colony-forming inhibition, two-fold serial dilutions of a sample of the cell suspension were made. From each dilution, cells were seeded into 60 mm petri dishes containing 4 ml of MEM. The resulting colonies were counted after incubation at 37°C for 6–7 days and staining by the May–Gründwald Giemsa technique.

Complement fixation

This was performed by a modification of the "chessboard" method of Bradstreet and Taylor [4], using 80-well plastic agglutination trays. Intact cells, as antigen, were used at various concentrations, starting at 10^5 cells/well. The initial dilution of antiserum was 1 : 10, as mouse serum is anticomplementary at higher concentrations. Three units of complement/well were used.

Control trays contained normal serum in place of specific antiserum or normal cells instead of polyoma virus transformed cells. Each tray carried control rows to test for anti-complementary action of either serum or antigen.

The trays were left to react overnight at 4°C, and then incubated at 37°C for 30 min after addition of 0·1 ml of 2% sensitized sheep erythocytes. Absence of haemolysis indicated fixation of complement.

RESULTS

Development of tumours in newborn mice and transplantability

Two separate experiments were carried out, the first in newborn F1 C3H/Bi × C57BL hybrid mice, the second in newborn C3H/Bi pure line mice. All mice received a single inoculation of 1×10^6 transformed non-malignant cells (7_2). Only those mice surviving longer than 5 days were taken into account.

Out of 97 hybrid mice inoculated intraperitoneally 39 (40%) showed tumours with a diameter of 1–3 cm after about a month. Of the 75 C3H/Bi mice inoculated either subcutaneously or intraperitoneally, 12 (16%) developed tumours 2–5 weeks after inoculation. Tumours were never observed when 10^5 or 10^4 transformed non-malignant cells were inoculated in 29 newborn C3H/Bi and 42 newborn F1 C3H/Bi × C57BL mice.

Some tumours were tested for transplantability. In four cases samples of the same tumours were used both for tissue culture and transplantation.

Each of 5 tumours from hybrid mice were transplanted into groups of 5 adult C3H/Bi, F1 C3H/Bi × C57BL, and C57BL mice; rapidly growing tumours developed in all but the C57BL recipients.

Suspensions of 8 tumours from C3H/Bi mice inoculated as newborn were transplanted into groups of 3–5 adult syngeneic mice; only 3 of these were transplantable.

Tissue cultures

To determine whether the transplantability of the tumours from newborn mice changed after growth *in vitro*, one of the transplantable tumours and three of the non-transplantable tumours were established in tissue culture.

When cells from these tissue culture lines were inoculated into groups of 5 adult C3H/Bi mice (10^6 cells/mouse), those from the transplantable tumour grew progressively whereas those from the non-transplantable tumours did not. Tests for transplantability of the malignant line (E20) were carried out at the 16th transfer generation and for the non-malignant line (E38) at the 29th transfer generation.

Tumourigenic titrations of the malignant cell line (E20) showed that $1 \times 10^{2.6}$ cells produced tumours in 50% of the animals.

Antigenic differences

In the colony inhibition test of Hellström and Sjögren, 2×10^4 cells were mixed with a 1 : 2 dilution of serum, prepared against the original line (7_2), and complement; the transplantable sub-line (E20) was only inhibited by 45%, whereas the non-transplantable sub-line (E38) was inhibited by 100%.

Using two-fold serial dilutions (1 : 4–1 : 256) of the serum prepared against the original line, (7_2), the 50% point of inhibition was $<1 : 4$ for the malignant line (E20) and 1 : 128 for the non-malignant line (E38).

The amount of complement fixed by the system (immune serum-intact transformed cell line E20 or E38) allows an indirect comparison of the amount of Py-specific antigen present on the surface of the cells. The malignant cells (E20) showed no detectable complement fixation whereas the non-malignant cells (E38) fixed complement at a serum dilution of 1 : 320. No complement fixation could be detected with this cell line when normal serum was substituted for immune serum.

DISCUSSION

In summary, the inoculation of the "transformed non-malignant" cell line (7_2) into newborn mice may be followed by tumour growth in a proportion of the animals. Some of these tumours were serially transplantable in adult syngeneic mice while others were not.

A different state of immunological competence may explain why the transformed, non-malignant cells were tumourigenic in newborn and not in adult mice: the cells would be accepted in the newborn because of their immunological tolerance, they would be rejected in the adults because of their immunological competance.

Some tumours from newborn mice were transplantable in adult syngeneic mice. It may seem plausible that lines of cells, genetically different from their parent cells, develop in the newborn during the tumour growth. One expression of this change would be the loss of sensitivity to cytotoxic antibodies. However it would be unjustified at this stage to identify this loss with malignancy. Thus we have confirmed *in vivo* findings previously observed *in vitro* [2]; intact animals as well as tissue culture allow the isolation of malignant cell variants, which have acquired *in vivo* a selective growth advantage.

We conclude that the different cells may be stages in the process of tumour progression. The observation of transformed cells which are antigenic and non-tumourigenic have been described in at least two other experimental tumour systems: for mouse cell cultures transformed by SV40 virus by Smith *et al.* [5], and Ehrlich ascites cell lines by Eng *et al.* [6]. This indicates that the phenomenon of immunological variation is not limited to our system.

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

Preferential Inhibition of Protein Synthesis in Normal or Neoplastic Tissues in Relation to Molecular Structure

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

Institute of General Pathology and C.N.R. Centre for Research in Cell Pathology, University of Milano, Italy

Abstract—*The* in vitro *study of kinetics of the incorporation of labelled amino acids into protein of normal and neoplastic tissues has demonstrated that* L-erythro-α,β-*dihydroxybutyraldehyde* (L-erythro-*DHBA*), *at concentrations which do not appreciably affect cell respiration, inhibits in a preferential way protein synthesis in tumours.*

In the presence of 5 *mM* L-erythro-*DHBA, the leucine incorporation into protein of a large number of tumours is completely inhibited during the* 2*nd hr of incubation. Under the same experimental conditions, the amino acid incorporation rate into protein of various normal tissues decreases as compared to controls, but it is never blocked. A relationship between the rate of protein synthesis and the sensitivity to the aldehyde has been observed in normal tissues but not in tumours. The tumours are affected by the drug independently of their histogenetic origin.*

L-erythro-*DHBA is not the only substance which acts preferentially on tumours. The comparative study of the effect of various* 3- *and* 4-*carbon aliphatic aldehydes on the* in vitro *leucine incorporation into protein of rat liver and Yoshida ascites hepatoma has demonstrated that* α-*hydroxybutyraldehyde,* β-*hydroxybutyraldehyde,* α,β-*dihydroxybutyraldehyde and its structural isomers are more active on the hepatoma than on the liver, while propionaldehyde, lactaldehyde, glyceraldehyde,* n-*butyraldehyde,* iso-*butyraldehyde, crotonaldehyde, and* α-*methylglyceraldehyde are more active on the liver than on the hepatoma. A complete inhibition of protein synthesis during the* 2*nd hr of incubation has been observed in the rat liver in the presence of* 5 *mM* iso-*butyraldehyde.*

On the whole, this data leads one to conclude that the branching of the carbon chain in the C_4-*aldehydes increases their selective toxicity for the liver, whereas the hydroxylation of the straight chain in the above-mentioned aldehydes gives rise to substances with a preferential action against tumours.*

ALIPHATIC aldehydes depress *in vitro* protein synthesis in normal tissues and in Yoshida ascites hepatoma cells bringing about a depletion of intracellular free cysteine and an impairment of the attachment of mRNA to ribosomes [1–4].

Among the aldehydes hitherto studied, DL-*erythro*, *threo*-DHBA* and L-*erythro*-DHBA were found to depress markedly *in vitro* protein synthesis in Yoshida ascites hepatoma cells at concentrations unaffecting tissue respiration and glycolysis [2]. These two compounds tested *in vivo* were shown to exert carcinolytic and carcinostatic activities in various experimental tumours [5, 6].

In an effort to understand better the mechanism of such activities, we studied the kinetics of *in vitro* leucine incorporation into protein of normal rat liver and rat hepatomas in the

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*DHBA = α,β-dihydroxybutyraldehyde.

presence of L-*erythro*-DHBA. In this preliminary research approach, L-*erythro*-DHBA turned out to be active in depressing protein synthesis much more in hepatomas than in normal liver. Then, we tested the *in vitro* effect of L-*erythro*-DHBA on the protein synthesis of various normal and neoplastic tissues. Finally, we investigated whether the molecular structure of 3- and 4-carbon aliphatic aldehydes could interfere with their biological activity.

The results of these investigations reported here show that various aldehydes depress protein synthesis more in neoplastic than in normal tissues, but others act in a quite different way. The striking fact is that these different biological behaviours are related to well defined molecular structures.

MATERIALS AND METHODS

Chemicals

Propionaldehyde, *n*-butyraldehyde, *iso*-butyraldehyde, and crotonaldehyde were purchased from B.D.H., Chemicals Ltd, Poole, England; DL-glyceraldehyde from Mann Research Lab., New York, U.S.A.; β-hydroxybutyraldehyde from Hopking and Williams, Chadwell Heath, England. Lactaldehyde, DHBA (DL-*erythro-threo*-; D-*threo*-; D-*erythro*-; L-*threo*-; L-*erythro*-) were synthesized by Laboratori Farmitalia, Milan, Italy. α-Methylglyceraldehyde and α-hydroxybutyraldehyde were synthesized in the Institute of Industrial Chemistry, University of Milan, Italy. Propionaldehyde, *n*-butyraldehyde, *iso*-butyraldehyde, β-hydroxybutyraldehyde, and crotonaldehyde were distilled before use. DL-Lactic acid (natrium salt) and DL-α-hydroxybutyric acid were purchased from Fluka A.G., Buchs SG, Switzerland, and *iso*-butyric acid from C. Erba, Milan, Italy.

L-Leucine-1-C^{14} was purchased from C.E.A., Gif-sur-Yvette, France; glycine-1-C^{14} from the Radiochemical Centre, Amersham, England; L-phenylalanine-C^{14}(U) from New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Unlabelled L-leucine, glycine, and L-phenylalanine were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Preparations of tissues

Normal tissues were obtained from albino Wistar rats, weighing 150–180 g, and from Swiss mice weighing 25–28 g, fed a commercial standard diet. Regenerating liver was used 3, 5 and 7 days after partial hepatectomy in albino Wistar rats according to Higgins and Anderson [7]. The animals were killed by decapitation, and the organs removed and sliced with a razor by hand. The diaphragm was divided into hemidiaphragms; the bone marrow from the femur and tibia of rats was collected pushing cold saline through the bone cavity. The rat embryos, whose age was evaluated on their crown-to-rump length [8], were sliced.

Ascites tumour cells were harvested from tumour-bearing animals 5–7 days after intraperitoneal transplantation of 0·5 ml of the ascites suspension. After dilution with ice-cold saline, the ascitic fluid was centrifuged for 5 min at 500 **g** at 2–4°C. The cells were then washed twice in cold saline and finally suspended in Krebs-Ringer phosphate. Samples of this suspension were taken for tissue dry weight determinations. Solid tumours were sliced as described for normal tissues, necrotic areas being avoided. Samples of human liver and tumour were from surgery.

Incubation procedures

Oxygen consumption was measured by standard manometric Warburg procedures. Tissue slices or cell suspension (8–12 mg of dry tissue) were incubated in 2·8 ml of Krebs-Ringer phosphate contained in the main compartment of the flask. The side arms contained either 0·2 ml of saline (controls) or 0·2 ml of aldehyde. The final osmolarity of the incubation medium was always adjusted to 0·30–0·31. A roll of filter paper absorbing 0·2 ml of 20% KOH was in the central well. Gas phase consisted of air; temperature was 37°C. After temperature equilibration for 10 min, the content of the side arm was tipped into the main compartment and readings were made every 10–15 min.

Protein synthesis was studied in a Dubnoff metabolic shaker at 37°C in an atmosphere of air. Tissue slices or cell suspension (8–12 mg dry weight) were incubated in Krebs-Ringer phosphate (final volume ml 3·0) containing 1·5 μmoles of labelled amino acid (specific activity, 0·266 mC/mmole) in the absence or in the presence of aliphatic aldehydes at different final concentrations. The final osmolarity of the incubation medium was always adjusted to 0·30–0·31. At fixed times, the tissues were treated with trichloracetic acid (final concentration 5%) and the precipitated proteins were purified according to Rabinowitz *et al.* [9]. Scintillation analysis was carried out with hyamine-treated samples to which 10 ml of scintillation fluid (4 g Omnifluor in a mixture of 700 ml toluene and 300 ml ethylene glycol-monoethylether) were added. Radioactivity

was measured in a Tri-Carb liquid scintillation spectrometer (Model 3365, Packard Instruments Co. Inc.) and corrected to 100% efficiency. On the basis of the radioactivity and of the specific activity, data were expressed as μmoles of amino acid incorporated/g protein according to Rabinowitz *et al.* [9].

RESULTS

The results of our initial investigation on the kinetics of the *in vitro* leucine incorporation into protein of normal rat liver and rat hepatomas in the presence of 5 mM L-*erythro*-DHBA are reported in Fig. 1. In normal liver the

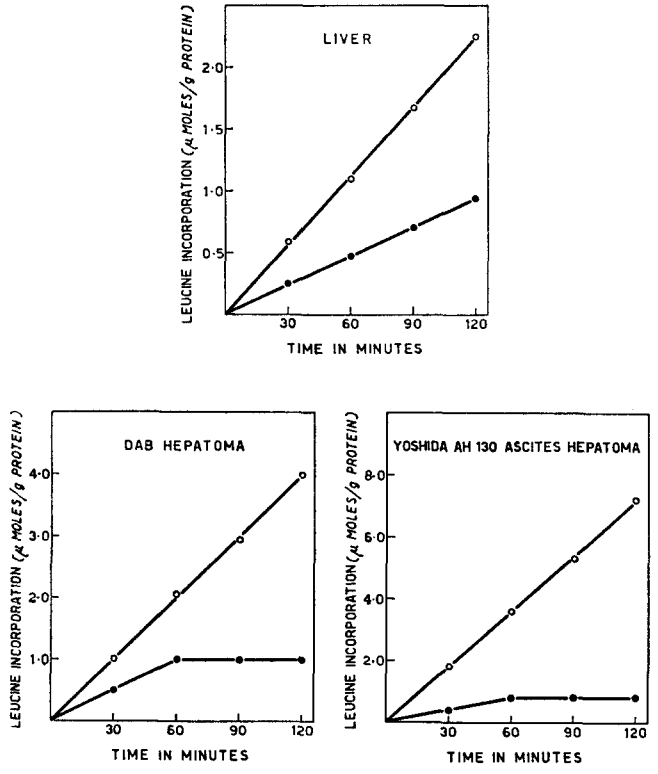

Fig. 1. *Effect of* L-erythro-*DHBA on the kinetics of leucine incorporation into protein of rat liver slices,* p-*dimethylaminoazobenzene (DAB) rat hepatoma slices and Yoshida ascites hepatoma cells. Tissues* (8–12 *mg, dry weight) were incubated at* 38°*C in Krebs-Ringer phosphate containing* 1·5 *μmoles of* L-*leucine*-1-C^{14} (*s.a.* 0·266 *mC/mmole*). *Medium volume,* 3 *ml. Gas phase, air. The final aldehyde concentration was* 5 *mM. Points are values of a typical experiment. Curves represent leucine incorporation into protein in the absence* (○) *and in the presence of aldehyde* (●)

Table 1. *Effect of* L-erythro-α,β-*dihydroxybutyraldehyde* (L-erythro-*DHBA*) *on the incorporation of some labelled amino acids into protein of rat liver slices and Yoshida AH* 130 *ascites hepatoma cells*

Amino acid	Tissue	Amino acid incorporation (μmoles/g protein)							
		Controls Time of incubation (min)				L-*erythro*-DHBA Time of incubation (min)			
		0–30	30–60	60–90	90–120	0–30	30–60	60–90	90–120
L-Leucine-1-C^{14}	Liver	0·70	0·74	0·70	0·68	0·35	0·39	0·33	0·37
	Hepatoma	1·18	0·94	1·00	1·06	0·76	0·15	0·06	0·00
	Hepatoma	1·21	1·31	1·55	1·46	0·59	0·12	0·00	0·00
Glycine-1-C^{14}	Liver	0·38	0·40	0·43	0·37	0·18	0·20	0·20	0·18
	Hepatoma	0·60	0·89	1·00	1·30	0·17	0·10	0·06	0·00
	Hepatoma	0·80	1·19	1·42	1·78	0·22	0·10	0·04	0·00
L-Phenylalanine-C^{14} (U)	Liver	1·08	0·99	1·25	0·93	0·48	0·44	0·44	0·46
	Hepatoma	0·63	0·71	0·82	0·75	0·32	0·11	0·07	0·00

Tissues (8–12 mg, dry weight) were incubated at 38°C in Krebs-Ringer phosphate containing 1·5 μmoles of labelled amino acid (s.a. 0·266 mC/mmole). Medium volume, 3 ml. Gas phase, air. The final concentration of L-*erythro*-DHBA was 5 mM. The results are values of single experiments.

amino acid incorporation rate in the presence of the aldehyde is inhibited up to a value of 50% but proceeds as a linear function of time like the control. In hepatomas, the leucine incorporation in the presence of aldehyde during the 1st hr of incubation is decreased by nearly 50% in DAB hepatoma slices and by nearly 77% in Yoshida ascites hepatoma cells as compared with controls; but during the 2nd hr, there is no further incorporation as graphically demonstrated by a plateau. The behaviour of this phenomenon both in normal and tumour tissues was confirmed using glycine-1-C^{14} or L-phenylalanine-C^{14}(U) as reported in Table 1. In a check for prolonged periods of time we observed no variation in shape of the above kinetics up to 4 hr of incubation.

The addition to the incubation medium of either glucose or succinate brings about an increase of the basal leucine incorporation into protein of Yoshida ascites hepatoma cells, but does not remove the inhibition of the protein synthesis by L-*erythro*-DHBA during the 2nd hr of incubation (Table 2).

Table 2. Effect of glucose and succinate on the in vitro *inhibition of leucine incorporation into protein of Yoshida AH* 130 *hepatoma cells by* L-erythro-α, β-*dihydroxybutyraldehyde* (L-erythro-*DHBA*)

Additions	Leucine incorporation (μmoles/g protein) Time of incubation (min) 0–30	30–60	60–90	90–120
Nil	1·52	1·60	1·45	1·57
Glucose	2·18	2·50	2·32	2·15
L-*erythro*-DHBA	0·52	0·38	0·00	0·00
Glucose + L-*erythro*-DHBA	0·72	0·57	0·04	0·00
Nil	1·75	1·90	2·05	1·75
Succinate	3·40	3·65	3·90	3·70
L-*erythro*-DHBA	0·60	0·42	0·00	0·00
Succinate + L-*erythro*-DHBA	0·92	0·60	0·02	0·00

Cells (8–12 mg, dry weight) were incubated at 38°C in Krebs-Ringer phosphate containing 1·5 μmoles of L-leucine-1-C^{14} (s.a. 0·266 mC/mmole). Medium volume, 3 ml. Gas phase, air. The final concentrations of glucose, Na-succinate, and L-*erythro*-DHBA were 5 mM. The results are values of two typical experiments.

All these data suggested that we verify whether the effect described above of L-*erythro*-DHBA is limited to the liver–hepatoma system of the rat or can also be observed in various normal and neoplastic tissues. The results reported in Table 3 show that L-*erythro*-DHBA acts in a similar way on the normal livers of rat, mouse and even man. In rat regenerating liver, bone marrow and embryo a reduction (but no blockage) of the protein synthesis occurs during the 2nd hr of incubation. In the case of the regenerating liver, the decreased leucine incorporation during the 2nd hr of incubation is nearly proportional to the mitotic index of the tissue.

The data reported in Table 4 confirmed that in tumours a total inhibition of protein synthesis occurs during the 2nd hr of incubation in the presence of 5 mM L-*erythro*-DHBA. In this connection we wish to point out that we examined different tumours including a hepatic metastasis of human gastric carcinoma, thus being able to present the following remarks. From our data a relationship between the growth rate of tumours and their sensitivity to the drug does not seem to be relevant. Indeed, the protein syntheses of the slow-growing Morris 7793 hepatoma and of the fast-growing Yoshida AH 130 ascites hepatoma are both blocked by L-*erythro*-DHBA. Furthermore, tumours of different histogenetic origin, as hepatoma, adenocarcinoma, epithelioma, carcinoma, carcinosarcoma, sarcoma, leukaemia and myeloma, in solid as well as in ascitic form, appear to exhibit a similar sensitivity to the drug.

The extent of this sensitivity with respect to normal tissues was studied with the following experimental approach. The leucine incorporation into protein after 2 hr of incubation was measured as a function of L-*erythro*-DHBA concentration in rat liver, bone marrow and

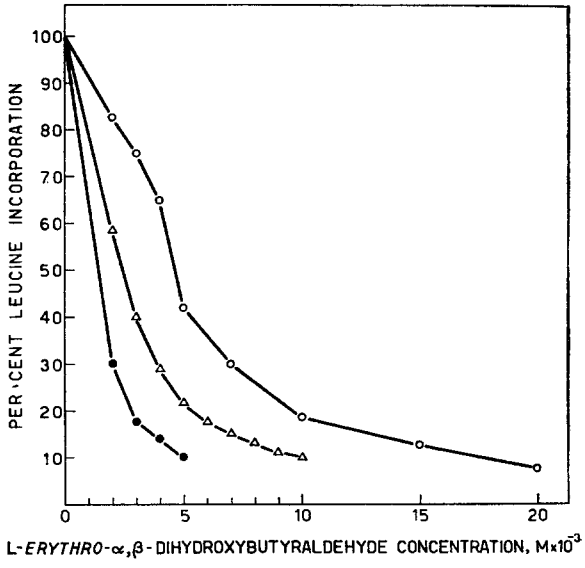

Fig. 2. Effect of various concentrations of L-erythro-*DHBA on the leucine incorporation into protein of rat liver slices* (○), *rat bone marrow cells* (△) *and Yoshida AH* 130 *ascites hepatoma cells* (●). *Tissues* (8–12 *mg, dry weight*) *were incubated for* 2 *hr at* 38°*C in Krebs-Ringer phosphate* (3·0 *ml*) *containing* 1·5 *μmoles of* L-*leucine*-1-C^{14} (*s.a.* 0·266 *mC/mmole*) *and the aldehyde as indicated* (*final concentration*). *The liver, bone marrow and hepatoma* (*without aldehyde*) *incorporated respectively* 2·66; 8·40; 9·36 *μmoles leucine/g protein during the* 2*hr incubation period.*

Table 3. In vitro *effect of* L-erythro-α,β-*dihydroxybutyraldehyde* (L-erythro-*DHBA*) *on leucine incorporation into protein of normal tissues*

Tissue	Leucine incorporation (μmoles/g protein)			
	Control		L-*erythro*-DHBA	
	Time of incubation (min)		Time of incubation (min)	
	0–60	60–120	0–60	60–120
Liver (rat)	1·20	1·10	0·52	0·42
Liver (mouse)	1·61	1·52	0·92	0·73
Liver (man)	1·72	1·54	1·02	0·83
Kidney (rat)	0·70	0·65	0·26	0·18
Spleen (rat)	1·91	1·74	1·14	1·30
Diaphragm (rat)	0·22	0·23	0·13	0·14
Testicle (rat)	1·53	0·98	1·20	0·84
Bone marrow (rat)	5·10	3·64	1·10	0·44
Embryo (14 days) (rat)	1·92	1·73	1·22	0·74
Regenerating liver (3 days) (rat)	1·71	1·44	0·83	0·44
Regenerating liver (5 days) (rat)	1·80	1·62	1·33	0·73
Regenerating liver (7 days) (rat)	0·92	0·84	0·50	0·44

Tissues (8–12 mg, dry weight) were incubated at 38°C in Krebs-Ringer phosphate containing 1·5 μmoles of L-leucine-1-C^{14} (s.a. 0·266 mC/mmole). Medium volume, 3 ml. Gas phase, air. The final concentration of L-*erythro*-DHBA was 5 mM. The results are mean values of two experiments. In the case of human liver, the results are values of a single experiment.

Table 4. In vitro *effect of* L-erythro-α,β-*dihydroxybutyraldehyde* (L-erythro-*DHBA*) *on leucine incorporation into protein of tumours*

Tumour	Leucine incorporation (μmoles/g protein)			
	Control		L-*erythro*-DHBA	
	Time of incubation (min)		Time of incubation (min)	
	0–60	60–120	0–60	60–120
Morris 7793 hepatoma (rat)	1·02	1·31	0·32	0·00
DAB hepatoma (rat)	1·63	1·64	0·78	0·00
Yoshida AH 130 hepatoma (rat)	1·72	1·63	0·52	0·00
Yoshida AH 130 ascites hepatoma (rat)	3·61	3·69	1·11	0·00
Walker 256 carcinosarcoma (rat)	1·99	1·32	0·78	0·00
Yoshida ascites sarcoma (rat)	2·00	0·91	1·02	0·00
Oberling–Guérin ascites myeloma (rat)	2·01	1·92	0·80	0·00
Ehrlich carcinoma (mouse)	1·92	2·11	0·42	0·00
Ehrlich ascites carcinoma (mouse)	5·04	3·64	1·52	0·00
Guerin epithelioma (mouse)	4·01	2·42	1·21	0·00
Lewis lung carcinoma (mouse)	2·22	1·94	1·02	0·00
Sarcoma 180 (mouse)	2·02	1·91	1·01	0·00
Sarcoma 180 ascites (mouse)	4·63	4·78	2·02	0·00
Sarcoma 45 (mouse)	7·40	6·01	3·30	0·00
Gross ascites leukaemia (mouse)	5·41	7·50	2·58	0·00
Gastric carcinoma (hepatic metastasis) (man)	5·20	4·90	2·90	0·00

The experimental conditions were as in Table 3. The results are mean values of two experiments. In the case of gastric carcinoma, the results are values of a single experiment.

Yoshida AH 130 ascites hepatoma. These materials belong to same animal species, and bone marrow is similar to tumours as far as its protein synthesis rate is concerned. The data of this study is reported in Fig. 2 where the percentage of incorporation was plotted against L-*erythro*-DHBA concentration. This demonstrates that in order to obtain in the bone marrow and in the liver the same extent of inhibition as observed in tumour, double and fourfold concentrations of L-*erythro*-DHBA respectively are required.

At this stage of our research, we were tempted to ascertain whether the above described effect is due to the aldehyde group, irrespective of the molecular structure, or whether the molecular structure may play a role in the protein synthesis inhibition. Our approach to such a programme

was to study the comparative action of a group of 3- and 4-carbon aliphatic aldehydes on the kinetics of protein synthesis in the rat liver–hepatoma system. These aldehydes were with straight or branched chains, saturated or unsaturated, unhydroxylated or hydroxylated in different positions as shown in Tables 5 and 6. To avoid the possibility that these compounds may act on protein synthesis by means of a general toxic mechanism depressing the energy supply, we previously tested the oxygen uptake of the tissues in the presence of drugs by selecting the concentrations which are practically uneffective on cell respiration, as shown in Table 5.

The results reported in Table 6 show that all the aldehydes tested depress leucine incorporation into protein of liver and hepatoma. This inhibition depends on the presence of the reactive —CHO group in the molecule since the corresponding acids (for instance lactic acid, *iso*-butyric acid, α-hydroxy- and β-hydroxybutyric acids) used at isomolar concentration do not affect protein synthesis. Moreover, the molecular structure of the aldehydes is important as far as the inhibition of protein synthesis in normal or neoplastic tissues is concerned. In fact, the data in Table 6 demonstrated surprisingly that the aldehydes act to a different degree on the liver and on the hepatoma. Propionaldehyde, lactaldehyde, *n*-butyraldehyde, *iso*-butyraldehyde, crotonaldehyde, and α-methylglyceraldehyde act preferentially on the liver. On the other hand, α-hydroxybutyraldehyde, β-hydroxybutyraldehyde, DL-*erythro-threo*-DHBA, D-*threo*-DHBA, L-*threo*-DHBA, D-*erythro*-DHBA, and L-*erythro*-DHBA act preferentially on the tumour. In the case of the hepatoma, an inhibition greater than 90% during the 2nd hr of incubation can be observed with β-hydroxybutyraldehyde, racemic DHBA and its four isomers.

The comparative effect of L-*erythro*-DHBA and *iso*-butyraldehyde on the leucine incorporation into protein of rat liver and hepatoma are given in Fig. 3. The data show that, under our experimental conditions, the two compounds act in oppositive ways, that is, *iso*-butyraldehyde completely inhibits protein synthesis in liver, but not in hepatoma, whereas L-*erythro*-DHBA completely inhibits protein synthesis in hepatoma, but not in liver.

DISCUSSION

In a series of studies reported previously the *in vitro* inhibitory effect of aliphatic aldehydes on protein synthesis was generally studied at a fixed time interval [1, 2]. To improve the information, as mentioned above, we extended our investigation to the kinetics of amino acid incorporation into protein of normal and neoplastic tissues in the presence of L-*erythro*-DHBA. This led to the observation that this aldehyde, in a wide range of concentrations,

Table 5. Effect of aliphatic aldehydes on oxygen consumption of rat liver slices and Yoshida AH 130 ascites hepatoma cells

Compound	Conc. (mM)	Oxygen consumption*			
		Liver		Hepatoma	
		Time of incubation (min)		Time of incubation (min)	
		0–60	60–120	0–60	60–120
Propionaldehyde	5	117	103	98	93
Lactaldehyde	5	96	86	91	93
DL-Glyceraldehyde	5	107	89	94	85
n-Butyraldehyde	5	129	102	113	113
iso-Butyraldehyde	5	133	96	113	113
Crotonaldehyde	0·25	96	95	103	111
α-Methylglyceraldehyde	5	98	98	94	87
α-Hydroxybutyraldehyde	5	109	106	86	88
β-Hydroxybutyraldehyde	5	108	75	89	64
	3	88	95	113	123
DL-*Erythro, threo*-α,β-dihydroxybutyraldehyde	5	102	90	94	95
D-*Threo*-α,β-dihydroxybutyraldehyde	5	98	96	92	90
L-*Threo*-α,β-dihydroxybutyraldehyde	5	97	95	93	92
D-*Erythro*-α,β-dihydroxybutyraldehyde	5	107	92	95	92
L-*Erythro*-α,β-dihydroxybutyraldehyde	5	115	93	94	96

*Compared to 100 in control experiments.
Tissues (8–12 mg, dry weight) were incubated at 38°C in Krebs-Ringer phosphate in the absence and in the presence of aldehyde. Medium volume, 3 ml. Gas phase, air. The results are mean values of two experiments.

Table 6. In vitro *effect of aliphatic aldehydes on leucine incorporation into protein of rat liver slices and Yoshida AH*130 *ascites hepatoma cells*

Compound	Conc. (mM)	Leucine incorporation (μmoles/g protein)			
		Liver		Hepatoma	
		Time of incubation (min)		Time of incubation (min)	
		0–60	60–120	0–60	60–120
Nil		1·52	1·23	2·92	2·88
Propionaldehyde	5	0·36	0·27	1·59	1·83
% Inhibition		76	78	46	37
Nil		1·42	1·54	5·60	5·84
Lactaldehyde	5	0·57	0·18	3·08	1·98
% Inhibition		59	88	45	66
Nil		0·81	0·79	5·03	5·18
DL-Glyceraldehyde	5	0·48	0·18	3·80	2·25
% Inhibition		41	77	25	57
Nil		1·73	1·58	3·75	2·90
n-Butyraldehyde	5	0·64	0·20	2·19	1·79
% Inhibition		63	87	42	38
Nil		1·61	0·88	3·80	3·98
iso-Butyraldehyde	5	0·70	0·02	2·07	2·06
% Inhibition		57	98	46	48
Nil		1·42	1·49	2·72	3·63
Crotonaldehyde	0·25	0·87	0·62	1·80	2·50
% Inhibition		39	58	34	31
Nil		1·28	1·20	4·59	4·69
α-Methylglyceraldehyde	5	0·68	0·68	4·15	4·00
% Inhibition		47	43	10	15
Nil		0·96	0·92	2·79	2·98
α-Hydroxybutyraldehyde	3	0·98	0·90	1·44	1·79
% Inhibition		0	0	50	40
α-Hydroxybutyraldehyde	5	0·62	0·59	0·91	0·75
% Inhibition		35	36	67	75
Nil		1·37	1·47	4·15	4·65
β-Hydroxybutyraldehyde	3	0·83	0·67	2·23	1·84
% Inhibition		39	54	46	60
β-Hydroxybutyraldehyde	5	0·54	0·32	1·12	0·07
% Inhibition		61	78	73	99
Nil		1·22	0·97	3·32	3·37
DL-*Erythro, threo*-α,β-dihydroxybutyraldehyde	5	0·72	0·44	0·98	0·29
% Inhibition		41	55	71	91
Nil		1·91	1·93	3·15	3·42
D-*Threo*-α,β-dihydroxybutyraldehyde	5	1·30	0·58	1·21	0·20
% Inhibition		32	70	62	94
Nil		1·52	1·38	3·72	3·77
L-*Threo*-α,β-dihydroxybutyraldehyde	5	0·90	0·43	1·38	0·16
% Inhibition		41	69	63	96
Nil		1·00	0·94	3·35	4·67
D-*Erythro*-α,β-dihydroxybutyraldehyde	5	0·68	0·30	0·85	0·04
% Inhibition		32	68	75	99
Nil		1·44	1·38	4·54	4·69
L-*Erythro*-α,β-dihydroxybutyraldehyde	3	1·05	0·95	1·20	0·18
% Inhibition		27	31	74	96
L-*Erythro*-α,β-dihydroxybutyraldehyde	5	0·74	0·70	1·08	0·00
% Inhibition		49	49	76	100

Tissues (8–12 mg, dry weight) were incubated at 38°C in Krebs-Ringer phosphate containing 1·5 μmoles of L-leucine-1-C^{14} (s.a. 0·266 mC/mmole) in the absence and in the presence of aldehyde. Medium volume, 3 ml. Gas phase, air. The results are mean values of two experiments.

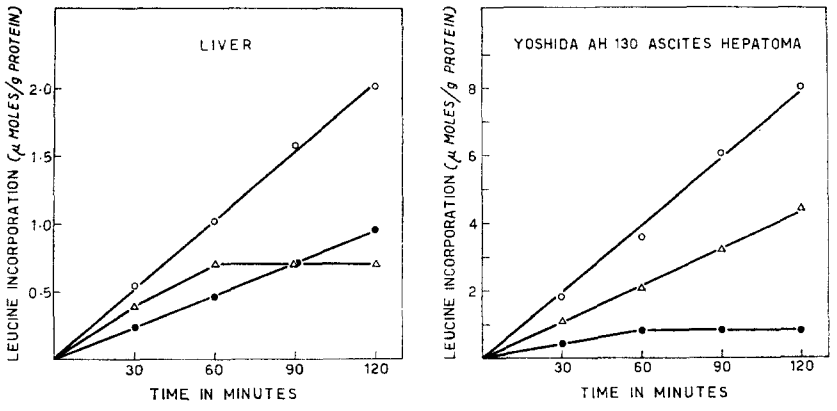

Fig. 3. *Effects of* iso-*butyraldehyde and* L-erythro-*DHBA on the kinetics of leucine incorporation into protein of rat liver slices and Yoshida ascites hepatoma cells. The experimental conditions were as in Fig.* 1. *The concentration of the aldehydes was* 5 *mM (final). Points are values of a typical experiment. Curves represent leucine incorporation into protein in the absence* (○) *and in the presence of* iso-*butyraldehyde* (△) *or* L-erythro-*DHBA* (●).

depresses protein synthesis much more in neoplastic than in normal tissues, *in vitro*. An almost selective antineoplastic activity appears evident when the kinetics of amino acid incorporation into protein is studied in the presence of 5 mM L-*erythro*-DHBA. Under these experimental conditions the protein synthesis in tumours is completely inhibited during the 2nd hr of incubation, while in normal tissues, although depressed as compared to controls, it generally follows a linear reaction up to the end of the incubation period.

A relationship between the protein synthesis rate and the sensitivity to L-*erythro*-DHBA was observed in normal tissues since rat bone marrow, embryo and regenerating liver are more affected by the drug than rat liver, kidney, diaphragm, testicle, and spleen. On the contrary, the inhibition of protein synthesis in tumours does not seem to be related to the rate of incorporation of labelled amino acid into protein. In fact in the presence of 5 mM L-*erythro*-DHBA, a complete inhibition during the 2nd hr of incubation was observed in tumours with a high rate of protein synthesis, as well as in tumours with a low protein synthesis rate. As to a comparison of sensitivity to the aldehyde between normal and neoplastic tissues, it should be noted that Yoshida ascites hepatoma is twice and four times as sensitive as rat bone marrow and liver, respectively.

The comparative investigation of the kinetics of amino acid incorporation into protein of a normal tissue and of a tumour originating from it (rat liver–hepatoma system) was applied to the study of the biological effect *in vitro* of small chain aliphatic aldehydes besides L-*erythro*-DHBA, in order to find out the importance of the molecular structure on the protein synthesis inhibition. Our data demonstrate that the presence of the reactive aldehyde group is indispensable for inhibition of protein synthesis and that the molecular structure plays an important role in determining the extent of this inhibition in normal and neoplastic tissues. The C_3-aldehydes (propionaldehyde, lactaldehyde, glyceraldehyde) do act more on the liver. Some C_4-aldehydes, as *n*-butyraldehyde, *iso*-butyraldehyde, crotonaldehyde, and α-methylglyceraldehyde are more effective on the liver, while others, as α-hydroxybutyraldehyde, β-hydroxybutyraldehyde, α,β-dihydroxybutyraldehyde and its stereoisomers are more effective on the hepatoma. These results show that the substitution of hydrogen atoms with hydroxylic groups in the molecule of *n*-butyraldehyde inverts the biological activity of this compound. As to the position of hydroxylic groups, it is interesting to note that β-hydroxybutyraldehyde has an antitumour activity higher than α-hydroxybutyraldehyde but, at the same time, it is more toxic than the latter on the liver; α,β-dihydroxybutyraldehyde and its isomers have a high antineoplastic activity and a liver toxicity between that of α-hydroxybutyraldehyde and β-hydroxybutyraldehyde.

It must be pointed out that branching of the carbon chain increases the selective toxicity on the liver. In fact in this tissue the protein synthesis is completely inhibited by a defined concentration of *iso*-butyraldehyde. The pre-

sence of two hydroxylic groups in the molecule of *iso*-butyraldehyde, as in the case of α-methyl-glyceraldehyde, reduces the toxicity without removing their preferential action on the liver. Therefore, the branching of the carbon chain seems to be more important than the hydrophilic or lipophilic character of the molecule in determining the hepato-affinity of C_4-aldehydes.

In conclusion, among the C_4-aldehydes it is possible to identify compounds acting *in vitro* preferentially either on the liver or on the hepatoma. The typical examples of these two classes of compounds are *iso*-butyraldehyde, which, at a defined concentration, completely inhibits protein synthesis in the liver but not in the hepatoma, and L-*erythro*-DHBA, which, at the same concentration, completely inhibits protein synthesis in the hepatoma but not in the liver. The opposite activity of these two compounds is most probably dependent on the different structure of the carbon chain as well as on the presence of hydroxylic groups in the straight chain. Both these molecular characteristics could be of importance in determining a difference (a) in the transport across the membrane, (b) in the metabolism, (c) in the affinity of the aldehyde to the protein synthesizing system, in the normal or neoplastic cell.

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The Effect of Diethylnitrosamine on the Distribution of Cell Classes in the Parenchyma of the Liver of Newborn Rats

L. WIEST

Institute for Cancer Research of the University of Vienna, Vienna, Austria

Abstract—*Newborn Sprague–Dawley rats were treated with daily doses of* 10 *mg/kg of diethylnitrosamine. The animals were sacrificed at different times after birth (up to* 30 *days), and the rates of DNA synthesis, the mitotic index and the relative amounts of binuclear and polyploid cells in the liver parenchyma were determined.*

The amount of tetraploid nuclei in the control rats increased to 12% *during the first month of life (the remaining* 88% *were diploid), whereas in the treated animals this value was already attained* 24 *hr after the first dose of DENA. In addition to this formation of tetraploid nuclei, still higher degrees of ploidy were found after prolonged treatment with the carcinogen. After* 5 *days,* 8n *nuclei were observed, after* 12 *days* 16n, *after* 22 *days* 32n *and after* 27 *days* 64n. *Approximately* 80% *of all parenchyma nuclei were polyploid after* 30 *days. During this time control rats showed only diploid and tetraploid nuclei.*

Binuclear cells amounted to 40% *between the* 20*th and* 40*th day in control animals, but did not exceed* 10% *in the DENA treated rats.*

From the 9*th day onwards there was no difference in DNA synthesis between these groups of animals.*

The two peaks of the mitotic index which are observed in untreated rats are not significantly present in the treated rats.

The conclusion can be drawn that diethylnitrosamine, in the concentration used, inhibits the amitotic and, to a lower degree, the mitotic division in the liver parenchyma which leads to an increased and earlier polyploidization of this tissue.

INTRODUCTION

THE PARENCHYMA of the liver of newborn rats is composed exclusively of mononuclear diploid cells. During the first 3 weeks of life other classes of cells develop, of which the percentages remain constant after the 10th week. The stationary amounts of the various types are: mononuclear diploid: 10%, binuclear diploid: 28%, mononuclear tetraploid: 53%, binuclear tetraploid: 2%, mononuclear octoploid: 7% [1, 2].

Hobik and Grundmann [3] treated adult rats with daily doses of 10 mg diethylnitrosamine (DENA) per 1000 g body weight until the appearance of liver tumours. During the precancerous phase an increase of the octoploid parenchyma nuclei could be observed and later even nuclei with the 16 or 32 fold chromosome number were found. Coté *et al.* [4] treated the animals with DENA doses half as high as those

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of Grundmann. They described an increased rate of DNA synthesis during the precancerous period while the mitotic index in the liver parenchyma was not altered by the carcinogen.

In this communication results are presented which describe the influence of DENA on the rates of DNA synthesis and mitosis and on the development of the different cell classes in the liver of young rats during the first month of life.

MATERIAL AND METHODS

Two hundred and forty Sprague–Dawley rats of both sexes were used. One hundred and thirteen animals received daily doses of 10 mg of DENA/kg body weight dissolved in isotonic sodium chloride solution by subcutaneous injection into the neck region. The control animals were injected with sodium chloride solution without the carcinogen.

Pieces of the right liver lobe were fixed in neutral 10% formaldehyde. These were cut into sections 14 μ thick and stained for DNA using the Feulgen method. The DNA content of individual nuclei was determined by microscope photometry (Reichert) at 560 mμ using the method of Swift [5]. The degree of ploidy of the nuclei was determined in this way by measuring 50 nuclei per animal.

Nuclear diameters were determined from 14 μ sections which were stained with haemalaune. One thousand nuclei were measured. The nuclear volumes were correlated to the DNA content and degree of ploidy.

The remaining part of each liver was immersed in a mixture of 8 parts Ca-free Locke's solution and 2 parts 0·1 M EDTA. After 2 hr the tissue and the solution were pressed through a syringe and stirred for 5 min. The resulting suspension of cells was washed twice with fresh Locke's solution [6]. Smears of this suspension were stained with haemalaune and the percentage of binuclear cells determined.

To 63 of the DENA treated animals and 88 control animals 0·5 μ Ci ^{3}H thymidine (sp.act. 5 Ci/mM) per g body weight was injected intraperitoneally and the animals were sacrificed 1 hr later.

The rate of DNA synthesis was determined by relating the incorporation of ^{3}H-thymidine to the DNA content of the liver. Aliquots of homogenated liver were used for DNA determination by the method of Burton [7] and for measuring the radioactivity by liquid scintillation counting (Packard Tri-Carb).

RESULTS

In order to determine the toxicity of DENA for newborn rats, we estimated the LD 50 value (lethal doses for 50% of the animals during the first 7 days). The value found, 210 mg/kg of body weight, corresponds to the value reported for adult rats [8].

Haematopoietic tissue

Numerous centers of blood cell production are present in the liver at the time of birth. These disappear between the 7th and 10th day in the control animals as well as in those treated with the carcinogen.

Cell classes of liver parenchyma

1. *Binuclear cells.* Twelve per cent of the liver parenchyma cells are binuclear up to 10 postnatal days. During the 4 weeks following this period the relative amount of these cells increases to 40% (Fig. 1). In the animals treated with DENA the amount of 5% present at birth was not altered until the end of the experiment.

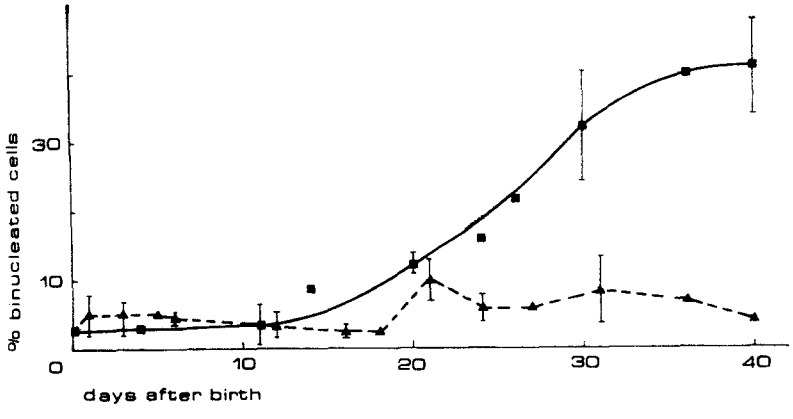

Fig. 1. Effect of diethylnitrosamine on the age-dependence of the percentage of binucleated cells in the liver parenchyma of rats.
▲------▲ *daily injection i.p. of 10 mg DENA per kg.*
■——■ *controls.*

2. *Mononuclear tetraploid cells.* The relative amount of tetraploid nuclei in the control rats increases from 2 to 12% during the first 40 days of life (Fig. 2). The other cells of the liver parenchyma are all diploid cells.

The treatment with DENA causes a rapid increase of the amount of tetraploid nuclei. After the first day we find 12% and after 18 days $39 \pm 2\%$ $4n$ nuclei. After this time the amount of tetraploid nuclei is decreasing again.

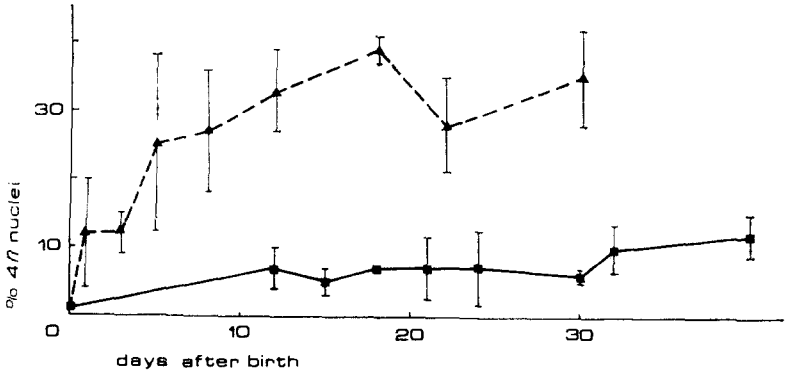

Fig. 2. Age dependence of the percentage of tetraploid nuclei in the liver parenchyme of rats treated with diethylnitrosamine
▲------▲ *daily injections i.p. of 10 mg DENA per kg.*
■——■ *controls.*

This is not caused by an increase in the relative number of diploid nuclei but can be fully attributed to a strong increase in the percentage of polyploid nuclei.

3. *Polyploid cells.* The nuclei of the liver parenchyma of control rats were diploid or tetraploid. Nuclei with a higher ploidy were never observed. In the animals treated with DENA we found octoploid nuclei after the 4th day, $16n$ nuclei after the 10th day, $32n$ nuclei after the 20th day and $64n$ nuclei after the 30th day. Intermediate levels of ploidy could also be observed (Fig. 3). After this time quantitative determination of the different nuclear classes was not possible any more because the first microtumours appeared and the liver parenchyma showed a very heterogeneous structure.

The mitotic index

In every animal 10,000 nuclei were counted and the number of mitoses determined. The mitotic index shows two age dependent maxima in the control animals. The first one occurs on the first day after birth and the second one after 22 days. After this second maximum the number of mitoses per 10,000 nuclei decreases regularly.

Treatment with DENA shifts the first maximum to the 4th day. A second peak of the mitotic index is observed on the 27th day being much lower than in the control animals.

DNA-synthesis

The results presented in Fig 5 show that the rate of DNA synthesis during the first week is

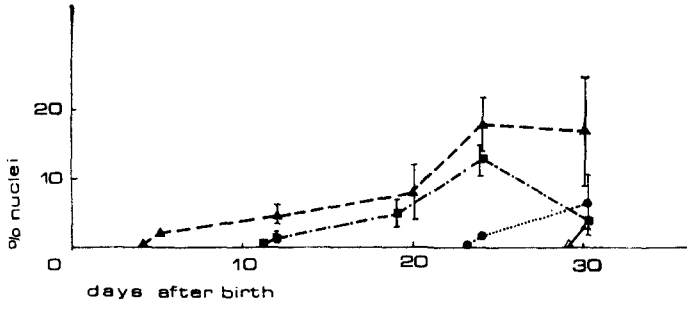

Fig. 3. Age dependence of the ploidy distribution in the liver parenchyme of rats treated with daily doses of 10 mg of DENA/kg.
▲------▲ 8n.
■-·-·-■ 16n.
●·····● 32n.
△——△ 64n.

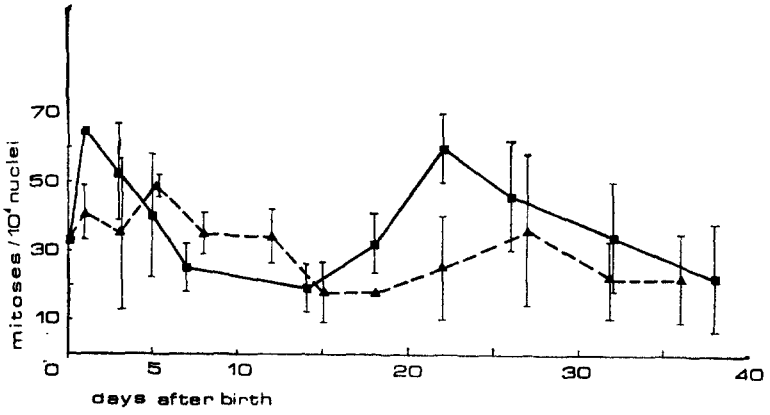

Fig. 4. Age dependence of the number of mitoses per 10,000 *liver nuclei.*
▲ - - - - - - ▲ *rats treated daily with* 10 *mg/kg of DENA.*
■ ——— ■ *controls.*

much higher in the controls than in the DENA treated animals. After this time no difference was observed.

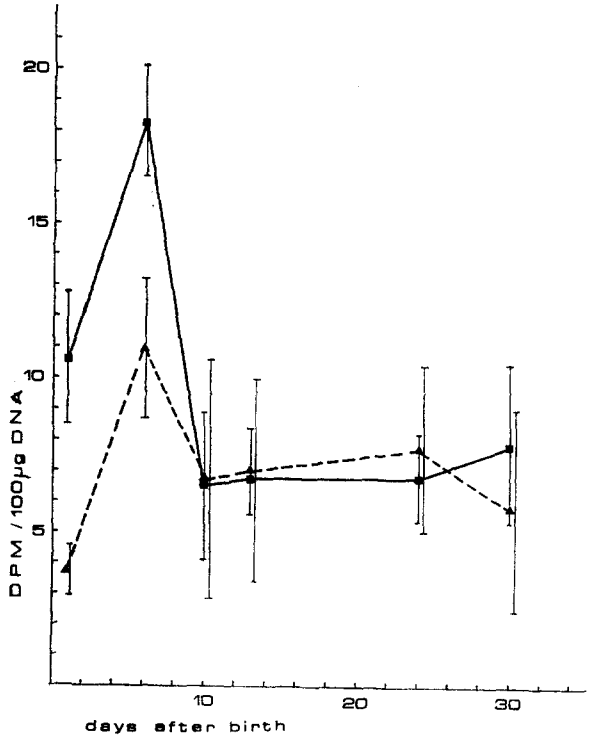

Fig. 5. Age dependence of the incorporation of ^{3}*H-thymidine into liver nuclei of rats treated with diethylnitrosamine* (10 *mg/kg/day*).
▲ - - - - - - ▲ *rats treated daily with* 10 *mg/kg of DENA.*
■ ——— ■ *controls.*

DISCUSSION

The cells of the liver parenchyma of newborn rats are mainly mononuclear and diploid. Tetraploid and binuclear cells are present only in amounts less than 5%.

Beginning with the 10th or 12th day various changes can be observed in the liver. The centers of blood cell production disappear while the mitotic index and the number of diploid and tetraploid nuclei increases.

Animals receiving from birth onward 10 mg/kg of diethylnitrosamine show distinct deviations from the normal sequence of events.

Already 24 hr after the first application of DENA the amount of tetraploid nuclei is increased to 12%. The high mitotic index which is found in this interval in the control animals does not occur in the DENA treated rats.

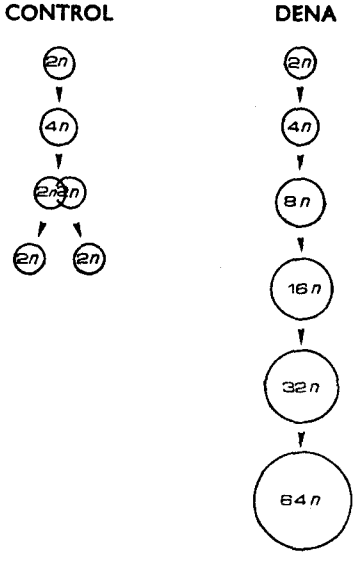

Fig. 6. Schematic representation of the development of cell classes in rat liver.

Controls: A cell with one diploid nucleus becomes endomitotically tetraploid. Subsequently the nucleus divides without concomitant cell division and a binuclear cell results. If cell division does occur two cells with one diploid nucleus each are formed.

Changes due to diethylnitrosamine: Sequential endomitotic steps result in the formation of 4n, 8n, 16n, 32n *and finally* 64n *nuclei. There is no nuclear division after endomitosis is completed.*

Furthermore nuclei with an 8-, 16-, 32- and even 64-fold chromosome number are formed. In comparison no nuclei larger than $4n$ are found in control animals.

The proportion of binuclear cells does not increase over 10%; this is 30% lower than in the control rats.

These results suggest that in untreated liver mononuclear tetraploid cells are formed by endomitosis from mononuclear diploid cells. After nuclear division these cells contain two diploid nuclei. Cell division follows immediately or is delayed (Fig. 6).

Treatment with the cancerogenic substance diethylnitrosamine prevents the division of tetraploid nuclei. Consequently octoploid nuclei are formed when another cycle of DNA-synthesis is completed. Repeated cycles of DNA synthesis result in the formation of nuclei with very high degrees of ploidy which cannot be reduced because nuclear division is hindered by the carcinogen.

This interpretation of the results implies that the binuclear cells are not the precursors of the tetraploid cells [9] but that the reverse takes place, so that the binculear cells originate from tetraploid mononuclear cells.

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Induction of Tumors in Newborn and Adult Swiss Mice by N-Diazoacetylglycine Amide

G. BRAMBILLA,* M. CAVANNA,* S. PARODI† and L. BALDINI†

**Department of Pharmacology of the University of Genoa, and †Department of Pharmacology and Pharmacognosy of the University of Trieste, Italy*

Abstract—*Short treatments with relatively high doses of N-diazoacetylglycine amide induced, in newborn and in adult Swiss mice, pulmonary tumors and lymphatic leukemia. The carcinogenic effect of this compound, which also exerts antineoplastic and immunodepressive activity, may be related to its alkylating action.*

INTRODUCTION

N-DIAZOACETYLGLYCINE amide (DGA) inhibits the growth of some transplanted tumors [1], and exerts immunodepressive activity [2]. On the basis of the well known reaction of aliphatic diazo-groups with acidic compounds [3], the biological activity of DGA has been attributed to its capacity to alkylate acidic cellular components.

Several alkylating agents are mutagens and carcinogens [4–6], and among the diazoalkanes the carcinogenic activity of diazomethane [7] and of diazoacetic ester [8] has been proved. In line with these findings we have tested the carcinogenic effect of DGA on newborn and adult mice.

MATERIAL AND METHODS

Random-bred Swiss albino mice of both sexes were used. Intraperitoneal injection of DGA was started either 48 to 72 hr after birth or at 30 to 35 days of age, and in both groups continued for 4 successive days. DGA was dissolved in saline just before use in a concentration that could be administered on the basis of 0·01 to 0·02 ml/g of body weight. Controls received i.p. 0·02 ml/g of 0·9% NaCl for 4 days, starting 48–72 hr after birth. In a few of the newborn mice there was a slight leakage of liquid where the needle was inserted.

Toxicity was estimated from the number of animals dead within 30 days after the end of the treatment. Those mice that were killed for abnormalities later or sacrificed at the end of the experiment (26–28 weeks after treatment) had complete necropsies. Tumor-like lesions were examined histologically, and used in the final accounting only when they showed to be tumors microscopically. Pulmonary tumors were counted by careful inspection of the entire surface of the lungs after fixation, and routine sectioning of all lungs were performed for assessing histologically their neoplastic nature.

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RESULTS

Table 1 summarizes the toxicity and the carcinogenic activity of DGA. Adult mice approximately tolerated a three-fold higher dose than newborns. Both in infant and in adult mice DGA induced pulmonary tumors and leukemia.

The incidence of pulmonary tumors in animals killed 26–28 weeks after the treatment was of 100%, independently of the dosage and of the age of the animals. Such tumors were not

Table 1. Toxicity and induction of leukemia and pulmonary tumors in newborn and adult Swiss mice given i.p. 4 successive daily injections of N-diazoacetylglycine amide

Age at beginning of treatment (days)	Dose (mg/kg/day)	Mortality*		Leukemia				Pulmonary tumors		
		No. dead/no. treated	(%)	Affected/examined mice	Incidence (%)	Latent period (weeks) Median	Range	Affected/examined mice	Incidence (%)	Mean nodule count†
2–3	Control (saline)	9/100	9	1/90	1·1	26	—	8/90	8·9	0·13
2–3	180	7/47	15	4/40[a]	10	16	14–18	40/40	100	38·3[c]
	300	9/59	15	16/37[a]	43	22	16–28	36/37	97	60·5[c]
	500	22/33	66	5/7	71	17	11–28	7/7	100	92·3
	833	17/19	89	2/2	100	19	—	2/2	100	—
	1388	7/7	100	—	—	—	—	—	—	—
30–35	500	0/33	0	9/26[b]	35	21	10–26	24/26	92	61·7[d]
	833	0/47	0	27/39[b]	69	16	11–26	38/39	97	94·9[d]
	1388	29/29	100	—	—	—	—	—	—	—

*30-day mortality.

†Average of all mice killed at 26–28 weeks after treatment.

[a,b]The frequencies of the groups marked with the same letter are significantly different ($P < 0·02$) between each other by X^2 test in a 2 × 2 contingency table, with Yate's correction.

[c,d]The means marked with the same letter are significantly different ($P < 0·05$) between each other by 't' test.

observed only in 4 mice killed for leukemia within 16 weeks after the treatment. A statistically significant increase in the average number of lung tumors per animal was observed by increasing the dose from 180 to 300 mg/kg/day in infant mice, and from 500 to 833 mg/kg/day in adult mice. In controls killed at 26 to 28 weeks of age the spontaneous incidence of such tumors was 8·9%, and in 7 of 8 mice single nodules were observed. Macroscopically, the tumors were rounded white nodules, 0·5–1·5 mm in dia., often localized just below the pleura. Microscopically, they were of adenomatous type; papillary formations were frequent in the larger tumors.

The incidence of lymphomas apparently followed a direct relationship to the dose. A statistically significant increase in the percentage of mice which developed leukemia was observed by increasing the dose from 180 to 300 mg/kg/day in babies, and from 500 to 833 mg/kg/day in adults. The average latent period, that ranged between 16 and 22 weeks, showed no correlation with the dose or the age of the mice. Among 90 controls killed at the age of 26 to 28 weeks we found only one leukemic mouse. In all the affected animals leukemia was of lymphocytic type and most of them showed enlargement of the thymus, spleen and lymph nodes. Microscopically, foci of leukemic infiltrates were found in liver, kidneys and lungs. Ascites occurred in 5 adult mice receiving 833 mg/kg/day.

The sex of the animals had no significant influence on the number of pulmonary tumors and on the incidence and latency of leukemia.

DISCUSSION

The data indicate that i.p. treatment with high doses of DGA induces pulmonary tumors and leukemia in a relatively short time, both in newborn and in adult Swiss random-bred albino mice. Extrapolation from the experimental data suggests that a significant incidence of pulmonary tumors could have ensued at lower dosages than those tested, since at all the administered doses it was already at 100%. On the other hand, for leukemogenic effect high levels of carcinogen were required. Both the incidence of leukemia and the average number of lung adenomas per animal were independent of sex. As regards a different susceptibility of adult and infant mice to tumor induction [9], clear conclusions cannot be drawn since the difference of toxicity precluded appropriate comparisons between groups treated with doses equivalent on the basis of body weight.

As far as the mechanism of the carcinogenic action of DGA is concerned, it seems reasonable to expect on the basis of its chemical structure that it is, in part or entirely, related to its alkylating activity. Extensive reviews on the chemical reactivity and carcinogenic properties of alkylating agents have been presented by Ross

[3], Walpole [6], and Brookes [10]. However, it is interesting to note that DGA exerts marked and long lasting immunodepressive effects [2], and this suggests the possibility that the compound might also act by inhibiting the processes by which mutant somatic cells are eliminated. Attention has been drawn by Berenbaum [11] and Green [12] to the connection between immunodepressive and carcinogenic activity. DGA might be a new example of such a correlation.

Since preliminary results of experiments now in progress show that neoplastic lesions of the same type are also induced by N-diazoacetylglycine hydrazide, the carcinogenic action appears to be a common property of derivatives of *N*-diazoacetylglycine, which share in various degree cytotoxic and antineoplastic activity.

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Plasma Dehydroepiandrosterone Sulphate Levels in Patients with Benign and Malignant Breast Disease

B. BROWNSEY, E. H. D. CAMERON, K. GRIFFITHS, E. N. GLEAVE,[1]*
A. P. M. FORREST[1]† and H. CAMPBELL[2]

Tenovus Institute for Cancer Research, Welsh National School of Medicine, Heath, Cardiff, Wales
[1]*Department of Surgery, Royal Infirmary, Cardiff, Wales*
[2]*Department of Medical Statistics, Welsh National School of Medicine, Royal Infirmary, Cardiff, Wales*

Abstract—*The plasma concentrations of dehydroepiandrosterone (DHA) sulphate and 11β-hydroxycorticosteroids (11-OHCS) were studied in groups of women with benign breast disease and those with primary and advanced breast cancer. Statistical analysis of the results obtained revealed that there was no difference in the levels of plasma 11-OHCS in the various groups of patients. However, patients with advanced breast cancer had lower plasma concentrations of DHA sulphate when compared to each of the other groups. The difference between the advanced and benign groups achieved significance* ($p < 0{\cdot}05$).

INTRODUCTION

IN THE last 10 years a number of groups [1–4] have shown that urinary 11-deoxy-17-oxosteroid excretion can be used to help discriminate between patients with breast cancer who will or will not benefit from adrenalectomy or hypophysectomy. Recently we have demonstrated that patients with advanced cancer of the breast, untreatable by local methods but requiringly administration of hormones, endocrine surgery or cytotoxic drugs, excrete significant lower levels of aetiocholanolone and androsterone than patients with benign breast diseases or primary breast cancer. This difference was found to be due to patients with advanced disease localized to the breasts, chest wall, regional lymph nodes, pleura or lymphangitis carcinomatosa and such patients affected the mean of the whole advanced group which also included patients with widespread metastasis to viscera, the skeleton and distant subcutaneous sites [5].

Current views suggest that the principal C_{19}-steroid synthesized by the human adrenal cortex is DHA sulphate [6] secreted in amounts of the order of 10–15 mg/24 hr (similar in quantity to that of cortisol) and this conjugate appears to be the major source of the 11-deoxy-17-oxosteroids found in the urine. However, conflicting reports have appeared which alternately suggest that women with advanced and primary malignant breast disease have either higher [7] or lower [8] plasma concentrations of 17-oxosteroids than their normal counterparts. Furthermore, Wang [9] has also reported that plasma DHA sulphate and androsterone sulphate levels in breast cancer patients are indistinguishable from those found in normal subjects. In view of this degree of uncertainty the present work was undertaken to re-examine the situation using a population of women collected from the South Wales area. Patients who were being treated for benign breast disease were taken as the best possible control

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*Present address: Department of Surgery, University Hospital of South Manchester, Manchester, M20 8LR.
†Present address: Department of Surgery, Royal Infirmary, Edinburgh.

group for comparison with those suffering from malignant disease.

PATIENTS AND METHODS

The levels of the plasma steroids were estimated in 83 women; 34 had benign breast disease; 23 had primary breast cancer; 26 had advanced localized or generalized cancer (21 advanced general, 5 advanced local).

Plasma and urine specimens

Plasma for the determination of steroid concentrations was separated from whole blood immediately after collection and this was stored in a frozen state until the estimations were done. All the patients included in the primary and advanced groups were in hospital and specimens were taken at a standard time (9.0 a.m.). Some of the patients in the benign group were in hopsital at the time of collection but some were out-patients.

Urine collections were obtained as described previously [5].

Adrenal stimulation test

Stimulation of the adrenal cortex was provoked with β^{1-25}-corticotrophin (Pentacosactride) kindly made available by Messrs. Sandoz Ltd. The dose given in each case was adjusted to the body weight of the patient (2·7 i.u./kg) This dose was calculated from a standard dose and average body weight. Basal samples of blood were taken at 9.00 a.m. and further samples at 3 and 4 hr, respectively, after administration of β^{1-25}-corticotrophin, which was given as a single intravenous injection immediately after the first venepuncture. Patients remained in a resting state until the test was completed. The plasma was separated in the manner described above and again stored in a frozen state until the steroid levels were measured.

Plasma 11-OHCS determination

Plasma 11-OHCS levels were estimated by a modification of the Mattingly [10] method. Fluorescence was determined by means of an Aminco Bowman Spectrophotofluorometer using a Xenon lamp in order to achieve greater specificity (excitation wave length 475 nm; emission wave length 535 nm).

Plasma DHA-sulphate determination

To each 4 ml plasma specimen 0·025 μCi [7α-^{3}H]DHA sulphate (Radiochemical Centre, Amersham, Bucks.; spec. act. 1000 mCi/mMol) was added in 500 μl ethanol to act as an internal standard for recovery estimation. Samples were then extracted twice with 4 volumes of ice-cold acetone and the pooled extracts reduced in volume to approximately 2 ml under vacuum (recovery 70·6% ±3·25). Following the addition of 20 ml methanol, fatty material was removed by extraction with an equal volume of 80–100°C light petroleum. The aqueous methanolic layer was again reduced in volume to approximately 2 ml, diluted with 10 ml distilled water, and extracted twice with 30 ml diethyl ether to remove unconjugated steroids. To the remaining aqueous phase was added $(NH_4)_2SO_4$ (6 g) and the conjugated steroid fraction was obtained by extraction with 3 × 30 ml diethyl ether : ethanol (3 : 1). Pooled extracts were taken to dryness on a rotary evaporator and the residue chromatographed on a thin layer of silica gel $HF_{254/366}$ (Merck, Darmstadt) in the system *tert*-butanol: ethyl acetate: 5N.NH_4OH (82 : 100 : 40, v/v). The area of silica gel containing the DHA sulphate was located by means of 10 μg standards run on the same plate and made visible as fluorescent spots under u.v. light (366 nm). The DHA sulphate area from the extract lane was then eluted by shaking the silica gel with 5 ml ethyl acetate and 3 ml saturated NaCl solution. Following a further 2 extractions with 5 ml volumes of ethyl acetate, the pooled extracts were reduced in volume to 10 ml. After the addition of 10 ml distilled water, 50 μl 16N.H_2SO_4 and 2 g NaCl, solvolysis [11] was carried out by shaking the mixture thoroughly and incubating at 50°C for 4 hr. After further shaking, the ethyl acetate layer was removed and the aqueous phase extracted once more with 10 ml ethyl acetate. The pooled extracts were then washed twice with 5 ml 5% $NaHCO_3$ solution and twice with 5 ml distilled water before being re-chromatographed on a thin layer of silica gel in the system *n*-hexane : ethyl acetate (1 : 1, v/v). When the appropriate portion of silica gel had been scraped off the plate into a test-tube, the DHA was eluted by addition of 2 ml distilled water and extraction with 3 × 5 ml benzene (final recovery, 38·7% ±11·30). The dried residue was re-dissolved in ethanol (1 ml) containing 1 μg 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) for subsequent use as an internal standard for gas chromatography. A portion of this solution (100 μl) was removed for determination of radioactivity (Mk I Nuclear Chicago Liquid Scintillation Spectrometer). The remainder was again taken to dryness, transferred to a 300 μl capacity glass stoppered test-tube and dried in a vacuum desiccator for 60–90 min at

room temperature. The 3-heptafluorobutyrate derivative was prepared [12] by dissolving the residue in 20 μl benzene and 20 μl heptafluorobutyric anhydride (Koch–Light, Colnbrook) and incubating the mixture at 65° for 30 min in a sand bath. Three standards of 0·5 μg, 1 μg and 2 μg DHA, each containing 1 μg of 5α-dihydrotestosterone were also prepared. When the reaction was complete, the reagents were removed by placing the tubes in a heated vacuum desiccator for exactly 2 min. The residues were then re-dissolved in 500 μl benzene and portions subjected to gas chromatographic analysis using a Pye 104 Chromatograph (Model 74—electron capture detector) on a column of 1% XE60 on Gas Chrom Q (Applied Science Laboratories, Philadelphia) at 185°C.

Urinary aetiocholanolone and androsterone determination

These measurements were performed as described previously [5].

RESULTS

The initial statistical analysis of the levels of plasma steroids showed that the absolute values of each were not normally distributed around their mean but had a markedly skew distribution. This is seen in Fig. 1a which shows the values of DHA sulphate for the patients with benign diseases. However, logarithmic conversion to the base 10 gave a unimodal and symmetrical distribution (Fig. 1b) and logarithmic values were therefore used in all further analysis. This finding was similar to our experience in a previous study of urinary steroid levels in similar groups of patients [5].

Comparison of the mean age of patients within each group shows no difference between the benign and advanced groups or between the primary and advanced groups (Table 1). However, there was a difference ($p < 0{\cdot}01$) in mean age between the benign and primary groups of 6 years.

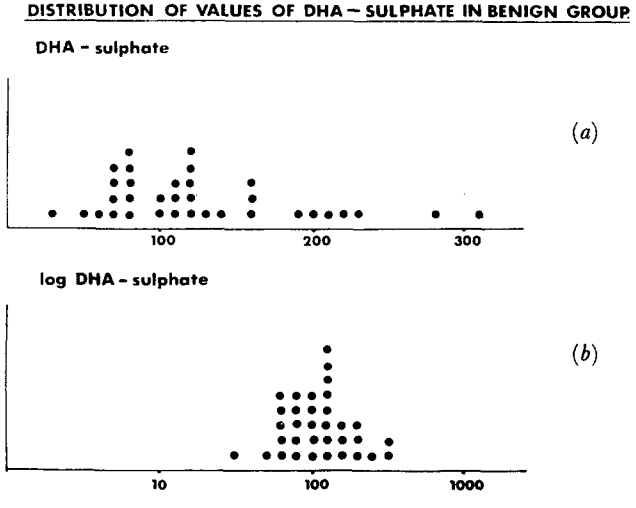

Fig. 1

Table 1. *Number of patients, mean ages and standard deviations, mean logarithmic values of cortisol and DHA-sulphate with standard deviations of benign, primary and advanced groups*

Group	No. of patients	Mean age ± standard deviation	Mean log value of cortisol ± standard deviation	Mean log value of DHA-S ± standard deviation
Benign	34	48·59 ± 6·16	1·44 ± 0·11	2·04 ± 0·22
Primary	23	54·17 ± 9·67	1·39 ± 0·19	1·92 ± 0·28
Advanced	26	51·69 ± 8·34	1·43 ± 0·19	1·77 ± 0·41

DHA sulphate

Since some patients with benign disease were in hospital at the time of collection of the plasma samples and others were out-patients, comparison of the mean logarithmic values of DHA sulphate for those sub-groups was made (Table 2) but no difference was found ($p < 0{\cdot}2$).

Comparison of the mean logarithmic values of DHA sulphate showed no difference between the benign and primary groups or between the primary and advanced groups (Table 1). There was however, a significant difference between the benign and advanced groups since patients with advanced breast cancer were found to have significantly lower levels of plasma DHA sulphate than the patients with benign breast diseases ($p < 0{\cdot}01$).

No correlation was found between the logarithmic values of DHA sulphate and age for any of the groups (Table 3). This may reflect the lack of sufficiently large numbers of patients in the earlier and later decades of life since the majority in the groups studied were between the ages of 40 and 60 years.

Table 2. Number of patients, mean logarithmic values of cortisol and DHA sulphate with standard deviations for in-patients and out-patients in the benign group

Group	No. of patients	Mean log value of cortisol ± standard deviation	Mean log value of DHA-S ± standard deviation
In-patients	13	1·42 ± 0·13	1·98 ± 0·22
Out-patients	21	1·46 ± 0·09	2·06 ± 0·21
Total	34	1·44 ± 0·11	2·04 ± 0·22

Table 3. Coefficients of correlation between logarithmic values of plasma DHA sulphate and age in benign, primary and advanced groups and the degree of significance

Group	Correlation coefficient (r)	Significance (p)	No. of patients
Benign	−0·1109	>0·1	34
Primary	−0·1967	>0·1	23
Advanced	−0·2812	>0·1	26

11-hydroxycorticosteroids

No difference ($p < 0{\cdot}3$) was found in the levels of plasma 11-OHCS between those patients with benign diseases who were in hospital at the time of collection or those who were out-patients (Table 2). Comparison of the mean logarithmic values of 11-OHCS for each group showed that there was no difference between them (Table 1).

Relation of urinary metabolites to plasma DHA sulphate

Twenty-two patients for whom plasma DHA sulphate was measured also had estimations of the 2 major urinary metabolites, aetiocholanolone and androsterone, performed. The coefficient of correlation between logarithmic values of the sum of aetiocholanolone and androsterone, and the logarithmic values of plasma DHA sulphate was calculated ($r = 0{\cdot}4344$). This relationship reached significance ($p < 0{\cdot}05$) (Table 4). These results are shown in Fig. 2 together with the calculated regression line.

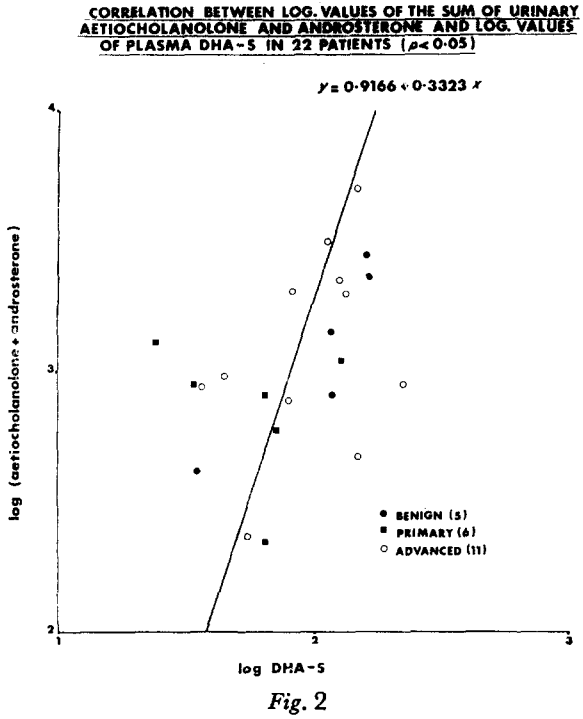

Fig. 2

Table 4. Coefficient of correlation between the logarithm of the sum of aetiocholanolone and androsterone and logarithmic values of plasma DHA sulphate in the benign, primary and advanced groups and the degrees of significance

Group	Correlation coefficient (r)	Significance (p)	No. of patients
Benign	0·8993	$<0·02$	5
Primary	−0·2365	$>0·1$	6
Advanced	0·3686	$>0·1$	11
Total	0·4344	$<0·05$	22

Adrenal stimulation with β^{1-25}-corticotrophin

The mean logarithmic values of plasma 11-OHCS and DHA sulphate for patients in all groups before and at 3 and 4 hr after the injection of β^{1-25}-corticotrophin are shown in Table 5. No differences were found between the groups for the mean logarithmic values of either steroid. The mean values of 11-OHCS in each group were above the upper limit of the normal range (5–25 μg%), suggesting that all patients were subject to some degree of stress. There was considerable difference in the levels of DHA sulphate in the patients studied both before and after injection of β^{1-25}-corticotrophin and the responses showed marked variability.

Table 5. Mean logarithmic values of cortisol and DHA-sulphate with standard deviations before and at 3 and 4 hr after β^{1-25}-corticotrophin in benign, primary and advanced groups

Group	No. of patients	Mean log values of cortisol ± standard deviation			Mean log values of DHA-S ± standard deviation		
		0 hr	3 hr	4 hr	0 hr	3 hr	4 hr
Benign	2	1·42 ± 0	1·75 ± 0·05	1·81 ± 0·08	1·65 ± 0·25	1·76 ± 0·21	1·74 ± 0·20
Primary	5	1·43 ± 0·07	1·81 ± 0·05	1·89 ± 0·13	1·64 ± 0·34	1·68 ± 0·38	1·74 ± 0·35
Advanced	8	1·47 ± 0·14	1·88 ± 0·10	1·95 ± 0·15	1·60 ± 0·33	1·70 ± 0·36	1·71 ± 0·31

DISCUSSION

No differences between the plasma concentrations of 11-OHCS or DHA sulphate were observed between those patients in the benign group who were in hospital at the time of collection of samples and those who were outpatients. This would indicate that hospitalization of patients does not provoke any greater 'stress' than attendance at a clinic as measureable by such parameters.

The lower concentrations of DHA sulphate in patients with advanced breast cancer might suggest that previous findings of lower urinary levels of aetiocholanolone and androsterone in such women simply reflects the concentrations of their plasma precursors. However, as Wang [9] has pointed out, care has to be exercised in drawing too close a parallel between plasma, DHA sulphate and urinary 11-deoxy-17- oxosteroid levels, since these metabolites can have a number of origins other than DHA sulphate, some of which may be of substantial quantitative importance. For example, the pool sizes and plasma concentrations of free DHA may be small but its metabolic clearance rate is very much greater than DHA sulphate [13, 14].

Although Wang [9] was unable to demonstrate a significant correlation between plasma DHA sulphate concentration and urinary 11-deoxy-17-oxosteroid excretion ($p = 0·1$) he stated that total plasma androsterone sulphate and DHA sulphate of patients with advanced breast cancer did show correlation with the urinary steroids. In contrast, the present study established that, in the series examined, plasma DHA sulphate concentration was correlated with the sum of its main urinary metabolites aetiocholanolone and androsterone ($p < 0·05$) (Table 4).

Earlier studies [5] revealed no difference in urinary 17-hydroxycorticosteroid excretion between various groups of patients. In the present investigations, no difference in plasma 11-OHCS concentrations were observed, again suggesting that this aspect of adrenal function is of little value in investigations into the aetiology of the disease (c.f. Wilson and Moore [3]).

The β^{1-25}-corticotrophin adrenal stimulation test also appeared to provide no useful information with regard to the natural history of the disease, but it was intriguing to note the apparent lack of consistent response to this ACTH preparation with respect to plasma DHA sulphate concentrations throughout the

population studied. No difference in plasma 11-OHCS and DHA sulphate concentrations between patients with benign breast disease and those with primary breast cancer could be detected. Similarly Wang [9] has reported that no differences could be observed in plasma DHA sulphate levels of normal women and women with early breast cancer. Indeed, he observed no differences between the latter groups and the group with advanced breast cancer. However, in view of the correlation found between plasma DHA sulphate concentration and urinary 11-deoxy-17-oxosteroid excretion, our observation of lower plasma DHA sulphate levels in patients with advanced breast cancer agrees with the significantly lower urinary 11-deoxy-17-oxosteroid levels detected in the advanced group [5] (particularly in those with advanced localized disease). Hence, this observation is compatible with the view that these levels also may be characteristic of the spread of the disease rather than its type. This picture does not entirely agree with that of Bulbrook *et al.* [1, 15] who showed that *both* advanced and early breast cancer groups had abnormally low urinary 11-deoxy-17-oxosteroid output. Sufficient data to demonstrate any statistically significant difference between the plasma DHA sulphate concentrations of those with localized and those with generalized spread of their disease are not yet available.

Thus, such differences as are detectable between groups of patients with breast disease remain small and overlap of results is extensive. In some respects, the days of accumulation of large series of hormone assay figures for statistical analysis may be numbered and valuable clues hitherto provided by such series should now be applied to more intensive investigations of much smaller numbers of individuals.

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Announcement

Symposium on Applications of Biomedical Technology

THE Symposium is to be held in Oxford from April 9th to April 12th, 1972 and will provide a forum for scientists and engineers with contributions to make to medicine and a higher standard of patient care. The symposium, held in the conference suite at the Headquarters of Pergamon Press Ltd., will be opened on Monday, April 10th by Sir Richard Doll, F.R.S., F.R.C.P., Regius Professor of Medicine, Oxford University. Throughout 2 days a wide range of papers will be presented by speakers drawn from branches of science, engineering and medicine. A choice of technical outings will also be provided. Delegates will be accommodated at St. Hilda's College, Oxford.

The conference fee will be £28 ($68) payable on registration and will include all conference sessions, accommodation, meals, transport if required, technical outings and two evening receptions. In addition a copy of a forthcoming publication by Pergamon Press entitled *Biomedical Technology in Hospital Diagnosis*, ed. A. T. Elder and D. W. Neill, provisional published price £13·50, will be donated to each delegate.

The conference secretaries are Dr. A. T. Elder, M.D., Ph.D., F.R.C.P., 2 Deramore Park, Belfast BT9 5JT, Northern Ireland, and Mr. A. Hendrie, 6 Selcourt Close, Woodley, Berkshire, England. Copies of the programme and registration forms may be obtained from Mrs. P. Ducker, Executive Secretary, Symposium on Biomedical Technology, Pergamon Press Limited, Headington Hill Hall, Oxford, England.

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Factors Influencing the Effect of an Adjuvant on the Growth of Solid Isogeneic Tumors

M. DONNER,* D. HOTTIER* and C. BURG

Unit of Experimental Cancerology and Radiobiology: U 95—*I.N.S.E.R.M., B.P.* 18, 54, *Vandoeuvre-lez-Nancy, France*

Abstract—*Experiments have been carried out in inbred mice to evaluate the effect of a* Proteus vulgaris *polysaccharide on the growth of solid tumors bearing specific antigens. In the case of a methylcholanthrene-induced sarcoma and an X-ray-induced adenocarcinoma, the tumor growth is enhanced when the bacterial product is inoculated, a few days before the tumor graft. By contrast, when* Proteus vulgaris *polysaccharide is injected, 2–3 weeks before grafting, the tumor development is delayed.*

Neutralization of tumor cells with serum shows that a soluble substance responsible for increasing of tumor growth is temporarily elaborated after the administration of polysaccharide.

It is suggested that polysaccharide alters the balance between humoral and cell-bound immune components and the results are discussed with respect to the presence of blocking antibodies.

INTRODUCTION

VARIOUS materials cause a non-specific stimulation of some immune responses and they are regarded as immunological adjuvants [1].

Numerous workers have investigated the effect of bacterial adjuvants on transplanted tumors, studying either the variations of tumor-takes, or survival time of tumor-bearing hosts.

It has been shown by Biozzi [2], Sanford [3] and Amiel [4, 5], that injection of B.C.G. or *Corynebacterium parvum* inhibited the development of ascites tumors (syngeneic and allogeneic).

However, the effect of bacterial agents on solid tumors is more debatable.

Halpern [6–8] and Old [9] found that inoculation of B.C.G., *Corynebacterium parvum*, or a bacterial extract exerted an inhibitory effect on the growth of non-specific tumors, such as Betz's Sarcoma, Sarcoma 180 and Carcinoma 755. Smith [10] and Woodruff [11] reported that an injection of *Corynebacterium parvum* delayed the appearance of transplants of a spontaneous mammary carcinoma and a methylcholanthrene induced sarcoma in isogeneic mice.

On the other hand, Old *et al.* [12] studied the effect of prior injection of B.C.G. on the growth rate of tumors recently induced with methylcholanthrene or benzopyrene. They reported that the growth of some tumors is significantly inhibited, whereas the development of other tumors is apparently enhanced.

Weiss *et al.* [13, 14] working with fractions of tubercle bacillus, observed that the growth rate of tumors could be either inhibited or enhanced.

This enhancement phemomenon has been also reported by Floersheim [15] with isologous lymphomas, polyomas and methylcholanthrene-induced sarcomas, after the inoculation of *Bacillus pertussis* vaccine.

Studying the tumor specific immune status of the primary tumor bearing host, Stjernswärd [16] observed that B.C.G. facilitated the takes of small isografts.

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*Attachée de recherches—I.N.S.E.R.M.

We previously tested the effect of B.C.G. and *Proteus vulgaris* extract on the immune response against tumor specific antigens and we reported that inoculation of these bacterial agents enhanced tumor growth [17, 18].

Although much information has been obtained, there have always been discrepancies among the results of different workers. At the present time, these anomalous results have not yet been fully explained and it can be asked if the timing of injections of bacterial products is critical.

To test this possibility, we have studied the effect of a *Proteus vulgaris* polysaccharide both on the first and the second set reactions against specific antigens of solid tumors, using variable time intervals between the treatment with bacterial extract and the tumor graft. The results of this study are presented in this report. On the other hand, we wanted to see whether the effect of polysaccharide is depending on soluble substances in serum of inoculated animals.

MATERIAL AND METHODS

1. *Animals and tumors*

The experiments were performed with 2 to 3 months old inbred mice of the C3H/He and XVII strains. Animals are maintained on a diet of standard mouse pellets and water *ad libitum*.

The experiments were carried out with a methylcholanthrene-induced sarcoma (CB1) in the C3H/He strain, and a radiation-induced adenocarcinoma (1095) in the XVII strain.

The transplantability of these tumors was examined through a series of TD50 assays. The TD50 is defined as that number of viable tumor cells which kill 50 % of a group of animals receiving each the same amount of viable tumor cells.

At the beginning of our investigation, the TD50 values in control and actively immunized mice in standard conditions were for the sarcoma CB1: control, 260 cells; immunized, 8×10^4 cells and for the tumor 1095: control, 8×10^3 cells; immunized, 21×10^3 cells. According to this criterion, sarcoma CB1 is highly antigenic and tumor 1095 weakly antigenic.

2. *Immunization*

A mouse bearing a tumor with a mean diameter around 15 mm is irradiated *in vivo* (8000 or 12,000 rads). Heavily-irradiated tumor fragments are minced and transplanted subcutaneously into the dorsum of the recipients.

Two weeks later, mice are immunized again in the same way. They are later challenged with viable tumor cells.

3. *Proteus vulgaris*

The polysaccharide of Proteus vulgaris was prepared as described previously [19] by a modification of the Westphal's method [20]. The endotoxin prepared by water–phenol method at 3 to 5°C, consists of lipopolysaccharide and proteinaceous material. The protein free lipopolysaccharide is isolated by using a further 50 % aqueous phenol extraction at 65°C. The species-specific polysaccharide is obtained from lipoplysaccharide by gentle hydrolysis with 0·1 N CH_3 COOH. The soluble fraction contains the polysaccharide and the insoluble one the lipid A component. Each animal was inoculated twice by the intraperitoneal route and received 100 μg (dry weight) of polysaccharide in physiological saline. This dose of polysaccharide did not itself produce any toxic symptoms. Control groups were treated with intraperitoneal injections of physiological saline in the manner used for polysaccharide treatment.

4. *Tumor cell suspensions*

Viable tumor cell suspensions are prepared by a modification of the method described by Boyse [21].

A tumor-bearing animal is killed and the tumor excised. All necrotic tissue is discarded and the tumor mass cut into small pieces with a pair of fine scissors. The pieces of tumor are placed in Hank's medium with trypsin and transferred to a plastic tube. The ends of the tube are joined and inserted in a peristaltic pump. The movement of the pump induces a circular displacement of the fluid inside the tube and ensures a progressive dispersal of the tumor cells. The suspenion is then cooled to 4°C, filtered through gauze, centrifuged twice and resuspended in Hank's medium. The viability of the tumor cells is determined with trypan blue stain and known numbers of viable cells in 0·2 ml volumes are injected subcutaneously into the dorsum of each mouse.

The animals are observed twice weekly for tumor development and survival time.

5. *Neutralization test*

To study the serum of polysaccharide-treated mice, experiments were performed, using a neutralization test.

Mice were immunized against sarcoma CB1 and treated with polysaccharide. The serum was collected either 7 or 20 days after the last injection of polysaccharide.

Mice were bled from the retro-orbital sinus. The blood was pooled and allowed to clot at room temperature. Twenty four hr later, the serum was collected by two centrifugations at 1000 **g** for 30 min.

Pools of sera from immunized and polysaccharide untreated mice and from normal mice were equally included in each experiment.

3×10^3 viable tumor cells of sarcoma CB1 were mixed with 2 ml of serum. After incubating at 37°C for 30 min, aliquots of each preparation were injected subcutaneously into normal C_3H mice.

RESULTS

Effect of time interval between pretreatment with Proteus vulgaris *polysaccharide and the tumor graft, on the growth of isologous solid tumors*

The data on survival time of C3H/He mice which received viable tumor cells of sarcoma CB1 are presented on Fig. 1. It is seen from this figure that 86% of the control mice die following the tumor graft, with a mean survival time of $56{\cdot}73 \pm 4{\cdot}23$* days.

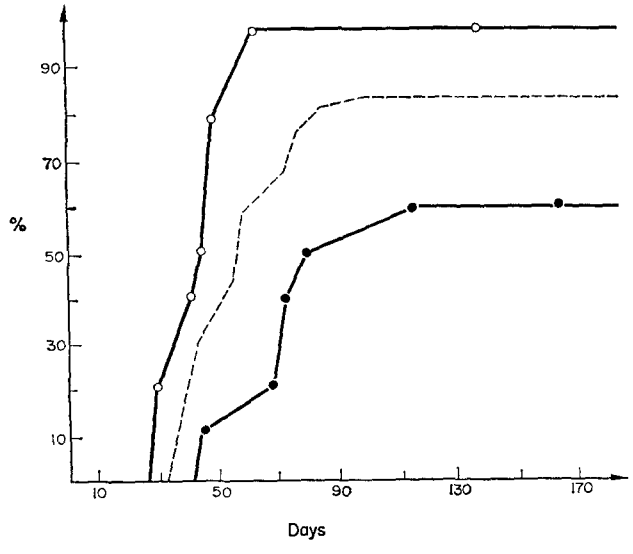


Fig. 1. Effect of timing of Proteus vulgaris injections on the lethality of mice C3H inoculated with cells of tumor CB1 Cumulative mortality (%) as a function of time since the day of grafting. - - - - - -, Control; ○——○, Proteus vulgaris, −5 and −1 day; ●——●, Proteus vulgaris, −19 and −14 days.

When *Proteus vulgaris* polysaccharide was administered, 5 days and 1 day before grafting, the tumor incidence increased. The mean survival time was significantly diminished: $44{\cdot}40 \pm 3{\cdot}47$* days (Student's t test $p < 0{\cdot}05$).

By contrast, pretreatment with *Proteus vulgaris*, 19 and 14 days before the tumor graft, inhibits the growth of tumors. Only 60% of mice are killed and the mean survival time ($74{\cdot}00 \pm 9{\cdot}22$ days) is significantly prolonged (Student's t test: $p < 0{\cdot}05$).

In analogous experiments, the effect of polysaccharide has been tested on the growth of adenocarcinoma 1095. The results are shown on Fig. 2.

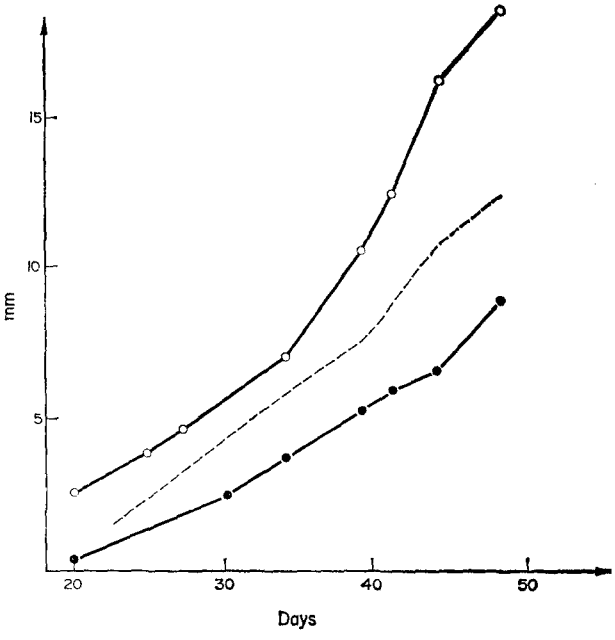


Fig. 2. Effect of time interval between the pretreatment with proteus and the tumor challenge on the development of an isogeneic adenocarcinoma (strain XVII) Mean tumor diameter (mm) as a function of time since the day of grafting. - - - - -, Control; ○——○, Proteus vulgaris, −5 and −1 day; ●——●, Proteus vulgaris, −35 and −30 days.

Figure 2 shows that the tumor isografts grow more rapidly in the group treated than in the control when *Proteus vulgaris* was given, a few days before grafting.

In contrast, the tumor growth is delayed when the mice received bacterial product one month before grafting of the tumor cells.

Effect of timing of Proteus vulgaris *injection on the growth of tumors in specifically immunized mice*

The animals are immunized by two inoculations of lethally irradiated tumor tissue, according to the schedule previously described.

The experimental designs are given in text (Fig. 3).

*Standard error.

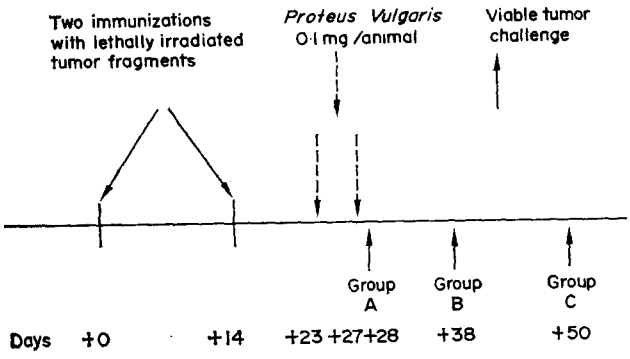


Fig. 3. Schematic representation of experimental conditions for the experiments A, B and C.

Animals received, 9 and 13 days after the second injection of irradiated tumor, 0·1 mg (dry weight) of *Proteus vulgaris* extract. They are then divided into three groups (A, B and C) and challenged with viable tumor cells, at various intervals after the second inoculation of *Proteus vulgaris*.

Together with each of these three groups, immunized controls which were not treated with *Proteus vulgaris* and unimmunized controls were included.

Table 1 shows the effect of *Proteus vulgaris* polysaccharide within each experiment A, B, and C. It is seen that the level of resistance in immunized hosts is subject to variations with the time. The tumor incidence undergoes fluctuations, owing to various time intervals between the last immunization and the challenge with viable tumor cells.

For these reasons, only animals immunized and challenged at exactly the same time can be compared with their control.

In experiment A, when *Proteus vulgaris* polysaccharide was administered one day before viable tumor cells, most of the mice are killed. The tumor incidence is significantly different between immunized mice treated and not treated with *Proteus vulgaris* ($\chi^2 = 10{\cdot}46$; $p < 0{\cdot}005$).

In these conditions, the resistance induced by immunization against viable tumor challenge disappeared after treatment with *Proteus vulgaris* polysaccharide.

In experiment B, when *Proteus vulgaris* was injected 10 days before grafting, no effect is observed. The tumor takes and the mean survival time of the tumor-bearing mice are not significantly different.

In contrast, when mice received *Proteus vulgaris* polysaccharide, 22 days before viable tumor cells, the tumor incidence is significantly diminished ($\chi^2 = 4{\cdot}08$; $p < 0{\cdot}05$). The mean survival time of the mice bearing tumors is increased (Student's t test = 3·08; $p < 0{\cdot}005$). The results obtained under these circumstances are the reverse of those observed in experiment A.

Variation of the neutralizing effect of serum towards tumor cells after polysaccharide treatment

Experiments were conducted to test the effectiveness of immune serum for cells of tumor CB1 after the injection of *Proteus vulgaris*

Table 1. Effect of timing of Proteus vulgaris *polysaccharide injections on the tumor growth in mice C3H immunized against tumor specific antigens of a methylcholanthrene-induced sarcoma*

Experiment	Pretreatment of animals	Days between *Proteus vulgaris* and the challenge with tumor cells	Number animals with a tumor/number animals tested	Tumor incidence (%)	χ^2* test	Mean survival time ± standard error (days)	Student's t test*
A	not immunized ———		53/55	96·5		41·40 ± 2·21	
	immunized ———		19/38	50·0	$\chi^2 = 10{\cdot}46$	43·83 ± 2·42	not
	immunized Proteus	1	34/36	94·5	$p < 0{\cdot}005$	41·14 ± 2·20	significant
B	not immunized ———		26/26	100		49·73 ± 3·03	
	immunized ———		16/27	64·0	not	58·00 ± 4·44	not
	immunized Proteus	10	29/47	61·7	significant	62·62 ± 3·61	significant
C	not immunized ———		30/30	100		49·55 ± 3·69	
	immunized ———		46/54	85·1	$\chi^2 = 4{\cdot}08$	46·86 ± 1·98	$t = 3{\cdot}08$
	immunized Proteus	22	36/54	66·6	$p < 0{\cdot}05$	59·55 ± 4·20	$p < 0{\cdot}005$

*χ^2 and Student's t test were calculated in the case of immunized mice, comparing the Proteus treated vs. the untreated animals.

polysaccharide. Tumor cells and serum were mixed and injected into mice according to the procedure described in "Material and Methods". The results of the neutralization test are presented in Figs. 4a and 4b. Immune serum of immunized and polysaccharide untreated mice exerts a slight inhibitory effect on the growth of tumors when comparing with normal serum. In contrast, the neutralizing action of immune serum is strongly altered when the animals receive polysaccharide. When the serum is collected 7 days after the last injection of polysaccharide, tumor growth is enhanced.

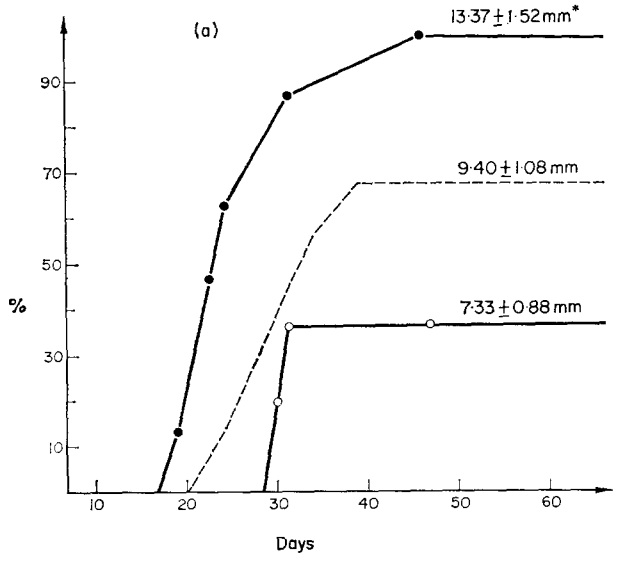
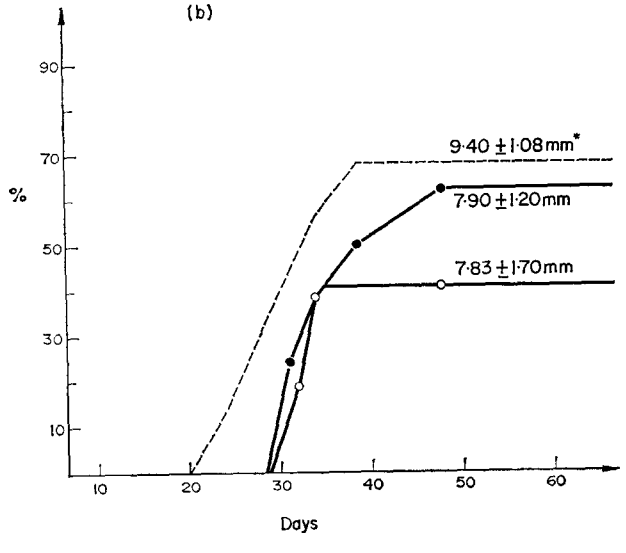


Fig. 4. Influence of polysaccharide of Proteus vulgaris on the neutralizing effect of serum towards tumor cells.
(a) The serum is collected 7 days after the last injection of polysaccharide.



(b) The serum is collected 20 days after the last injection of polysaccharide.
○——○, Tumor cells mixed with serum of immunized and polysaccharide untreated mice; ●——●, Tumor cells mixed with serum of immunized and polysaccharide treated mice; - - - - - normal serum. Ordinate: Tumor incidence in %. Abscissa: Days after the graft.
**Mean tumor diameter, on 60th day, in mm ± standard error.*

All mice which were injected with cells mixed with this serum display tumors. The data in Fig. 4a show that the tumors are better grown in this group than in the control one injected with cells and normal serum. The mean tumor diameter is equally increased: control, 9·4 ± 1·0 mm; experimental, 13·3 ± 1·5 mm, (Student's t test $p < 0{\cdot}05$).

This enhancing effect of serum of polysaccharide treated mice diminished when it is collected 20 days after the last injection of polysaccharide. The incidence and the dimensions of tumors are slightly increased in comparison with the group which received cells and immune serum of polysaccharide untreated mice. The results in Fig. 4b do not present significant differences.

DISCUSSION

From the above results, it is apparent that a polysaccharide of *Proteus vulgaris* has a dual effect on the interaction of an organism with a transplanted tumor. When bacterial extract is administered 3 to 4 weeks before grafting, it inhibits the growth of transplants of isologous solid tumors. By contrast, injection of polysaccharide, a few days before tumor cells, increases the tumor incidence. Furthermore, when an immunity is previously induced against tumor specific antigens, the administration of *Proteus vulgaris* polysaccharide provides comparable effects.

On the other hand, experiments using the neutralization of tumor cells with serum seem to indicate that the increasing of tumor growth is due to a soluble substance which is elaborated after the administration of polysaccharide.

In the present state of our investigation, there are several mechanisms to explain our results. However, it is obvious that all these explanations present a hypothetical aspect, since the mode of action of adjuvants is still poorly understood.

The fact that the polysaccharide of *Proteus vulgaris* inhibits the growth of transplanted tumors, is presumably on the line of observations reported with agents such as B.C.G., *Corynebacterium parvum, Brucella abortus*. These agents cause a non-specific stimulation of the immune system and increase the rejection of transplanted tumors. Working in another experimental system, Mizuno [22] reported an antitumor

effect of a lipopolysaccharide of *Proteus vulgaris* on Sarcoma 180 and Ehrlich carcinoma cells.

Nevertheless, the fact that the administration of polysaccharide induces in some cases an increasing of the tumor incidence linked with the temporary elaboration of a soluble substance requires more comments.

A first possibility is that *Proteus vulgaris* polysaccharide reduces immune response against tumor associated antigens by antigenic competition. Indeed, Radovich and Talmage [23] theorized that the antigenic competition is due to a humoral factor. More recently, Gershon [24] reported results which support their conclusions and extend them by suggesting that the Thymus-derived cells elaborate this humoral factor. If this is correct, it is conceivable that this humoral factor is not a conventional antibody, since the T cells do not secrete detectable quantities of antibody.

At present, the nature of the soluble substance responsible in our study for increasing of tumor incidence is unknown. Further experiments are necessary for providing some information on this factor. However, it is a reasonable speculation that the substance may be an antibody. For these reasons, our results suggest that the phenomenon of antigenic competition can be excluded.

A more probable explanation is that our findings are due to a classical phenomenon of immunological enhancement [25]. Indeed, a transplant of cells or tissues induces in his host a series of complex events which determine, either the acceptance or the rejection of the graft. It is known that the fate of tumor isografts is dependant on interaction between two antagonistic immunological reactions: a cell-mediated cytotoxic effect and an enhancement phenomenon linked with humoral antibodies. Among the different mechanisms advanced to explain this phenomenon, it is suggested that the coating of tumor cells with antibody prevents access of lymphocytes and allows the growth of tumors. In our experimental system, it is conceivable that the administration of polysaccharide changes the kinetics of cell-mediated and humoral immune reactions and causes an early and increased production of circulating antibodies. If this is correct, our results suggest that the production of these blocking antibodies rapidly declines. Subsequently, the cell-mediated immune response might be increased and would favor rejection of tumors.

Further analysis of other bacterial extracts may provide additional information, for it is possible that all adjuvants do not present the same mode of action on the tumor development.

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Causes of Death in Patients with Cancer

JEAN KLASTERSKY, DIDIER DANEAU and ALAIN VERHEST

Service de Médecine et Laboratoire d'Investigation Clinique (Section des Maladies Infectieuses), et Service d'Amatomie Pathologique, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Bruxelles, Belgique

Abstract—*The causes of death were analysed in 157 patients who died at the Institut Jules Bordet and a complete anatomic examination was performed on each patient. The clinical and bacteriological data available were taken into consideration, as well as the findings at autopsy, but only overwhelming terminal events were considered.*

Infection caused by gram-negative microorganisms was the most frequent cause of death in this series and accounted alone for 31·8% of the deaths. Severe haemorrhage was a significant cause of mortality in patients with acute leukaemia but ranked far behind infection. Neoplastic extension to vital organs was a significant cause of death in patients with cancer of the breast and cancer of the lung.

Although 90% of the patients in this series had very extensive tumoral spread beyond any possibility of cure by today's antineoplastic therapy, it is believed that supportive care, aimed at the prevention and treatment of infection in such patients might improve their chance for longer survival.

INTRODUCTION

RECENT improvements in the therapy of patients with cancer by new cytostatic agents and combination chemotherapy programmes can significantly alter the natural history of many malignant diseases. Therefore periodic evaluation of the causes of death in patients admitted to centres specialising in cancer therapy may provide guidelines relative to areas where further research on supportive care in malignant diseases is needed.

The fact that bacterial infection is the major cause of death in acute leukaemia has been stressed by Hersch and co-workers [1], but similar studies in patients with cancer in general have been less significant and no recent reference in this field is to be found. Our evaluation was undertaken to examine the causes of death in patients admitted to a centre for cancer therapy where modern and aggressive treatment is currently performed. Special emphasis has been put on infection as a cause of death in such patients. It should be noted, however, that only overwhelming terminal events have been taken into consideration. The importance of non-fatal but often serious infection and haemorrhage in patients with cancer is not reflected in our results.

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Please address requests for reprints to Dr. Jean Klastersky, Service de Médecine, Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium.

MATERIAL AND METHODS

The charts of 157 patients, with histologically proven cancer, who died and had a complete post mortem examination performed at the Institut Jules Bordet over a period of 18 months (January 1970–June 1971) were reviewed. The clinical and pathological causes of death were analysed, and the final cause of death determined. Only pathological states sufficient to cause death alone or in combination were recorded, excluding minor degrees of infection, haemorrhage or other morbid conditions.

Clinical signs and symptoms of severe infection were required to consider sepsis as a cause of death. Septicemia was diagnosed if positive blood cultures were obtained during

the last 7 days of life or if positive heart blood was obtained at autopsy. For pneumonia clinical features of a respiratory death were required as well as bacteriological evidence of pulmonary bacterial pathogen within the sputum or the lung parenchyma before death or at autopsy respectively. Severe local infection in the kidneys, peritoneum or soft tissue was also considered as a cause of death when it was associated with symptoms of generalized infection and terminal vasomotor collapse that could not be explained by other causes such as haemorrhage, pulmonary and myocardial infarction or extensive neoplastic infiltration of vital organs. To be considered as a cause of death, haemorrhage had to be extensive or in a vital organ. Death was attributed to the malignant process itself in cases where extensive metastases to the brain, liver or skeleton were responsible for coma, failure of liver function or intractable hypercalcemia, without evidence of life-threatening infection or haemorrhage.

Only treatment given during the last hospital stay was considered in the evaluation of the role of therapy in the causes of death. Full therapeutic doses had to have been given for at least 1 week.

In this laboratory the sensitivity of pathogenic microorganisms is routinely tested by the inocula-replicating method of Steers and coworkers [2]. The susceptibility of the organisms responsible for significant infection in this series was reviewed in an attempt to define which antibiotics might be considered as potentially life-saving drugs in severe infections in patients with extensive malignant diseases.

RESULTS

This study concerns 157 patients who died over a period of 18 months at the Institut Jules Bordet. The various underlying neoplasms found in these patients are shown in Table 1, and their distribution presumably reflects the major interests in cancer therapy in our hospital. Among these 157 patients only 16 (10%) were shown at autopsy to present a malignant disease that could have been cured, at least theoretically, by radiation or surgical therapy. Infection was the single predominant cause of death in these patients and was found in 7 of them. All the other patients presented extensive metastatic disease far beyond any available curative therapy.

In 7 (4·4%) cases no definite cause of death could be found: death occurred unexpectedly. No particular type of neoplasia could be demonstrated in these patients; but all of them presented very extensive malignant disease at autopsy.

Table 1. Types of cancer found in 157 consecutive patients who died at the Institut Jules Bordet

Leukaemia	27	
Acute lymphoblastic		8
Acute myeloblastic		11
Acute monoblastic		2
Chronic lymphatic		5
Chronic myelogenous		1
Lymphoma	20	
Lymphosarcoma		7
Reticulum cell sarcoma		8
Hodgkin's disease		5
Lung	26	
Breast	22	
Upper respiratory and digestive tracts	22	
Oesophagus		1
Oral cavity and pharynx		7
Larynx		14
Gastro-intestinal and genito-urinary tracts	24	
Pancreas		3
Rectum		6
Bladder		4
Kidney		3
Ovary		2
Uterus		1
Cervix		3
Vulva		1
Testicle		1
Other	16	
Thyroid		6
Melanoma		2
Skin		4
Fibrosarcoma		1
Myosarcoma		2
Parotid		1

As shown in Table 2, infection alone was the cause of death in 50 (31·8%) of the patients in this series, and was associated with another fatal process in 22 (14%) other cases.

Severe haemorrhage was responsible for death in 18 (11·4%) cases and was associated to another cause in 8 additional patients.

In 31 (19·7%) cases the neoplastic extension to the brain, lungs, liver or skeleton had caused, in the absence of a haemorrhage or infection, lesions that were considered sufficient to explain death.

Infection

In 72 patients infection was the only or predominant cause of death. The types of infection and the microorganisms responsible for it are shown in Table 3. Pulmonary infection

Table 2. Causes of death observed in 157 consecutive patients

	N°	%
Infection	50	31·8
Haemorrhage	18	11·4
Metastases		
—cerebral	10	6·3
—pulmonary	9	5·7
—hepatic	8	5·1
—osseous	4	2·5
Cardiac failure or infarction	7	4·4
Pulmonary embolus	5	3·1
Pulmonary fibrosis*	2	1·2
Pericarditis*	3	1·9
Hepatitis†	3	1·9
After lumbar puncture	1	0·6
Thrombosis of carotid or cerebral vessels	6	3·8
Infection		
+ haemorrhage	6	3·8
+ cerebral metastases	6	3·8
+ pulmonary metastases	3	1·9
+ hepatic metastases	1	0·6
+ cardiac failure	1	0·6
+ pulmonary embolus	2	1·2
+ pancreatitis	1	0·6
Haemorrhage		
+ cardiac failure	2	1·2
Infection		
+ cerebral metastases		
+ pulmonary embolus	2	1·2
Unknown	7	4·4
Total	157	100

*As a result of radiation therapy.
†No toxic or infectious causes were found.

was the most frequent type of infection in this series and septicemia originated most often from pulmonary sites of infection: 10(47·6%) out of 21 cases.

In 7 cases several microorganisms were involved in the infection that was considered to be associated with a fatal outcome. Sixty episodes (76%) of infections were caused by gram-negative bacilli (24 Klebsiella, 8 Pseudomonas aeruginosa, 14 *E. coli*, and 14 Proteus); this high incidence of infections caused by gram-negative microorganisms is in accordance with observations made by other investigators [3], suggesting that infections caused by gram-negative rods are the most frequent cause of infection in hospitalized patients at the present time. Staphylococci were responsible for 10 (12·8%) serious infections in this series. The sensitivity of gram-negative organisms responsible for serious infections in this series was tested by the inocula-replicating method [2] using an inoculum containing 10^2 microorganisms/ml. The pattern of resistance was similar to that reported recently from this laboratory [4]. It should be emphasized that none of the commonly used antibiotics, with the exception of gentamicin can provide an adequate cover for all the gram-negative rods responsible for serious infections in patients with malignant diseases. Fungal infections were found in 4 patients: generalized candidiasis was found in one patient with acute leukaemia and diffuse pneumonia caused by candida

Table 3. Most common types of infection and microorganisms responsible for them

	Pneumo-coccus	Staphy-lococcus	Klebsiella	Pseudo-monas	*E. coli*	Proteus	Viruses	Fungi	Myco-bacteria
Cases where infection was the only cause of death									
Septicemia		2	8	1	6	1*	1		
Pulmonary infection	1	3	7	3	3	2	1	1	
Urinary infection			1	1	1	1			
Wound infection			1			2			
Peritonitis		1*			2	3*			
Cases where infection was a significant cause of death									
Septicemia					1			1	
Pulmonary infection		3	6	2		1		2	1*
Urinary infection						1			
Wound infection						2	1*		
Peritonitis		1*	1*	1	1	1			
Total	1	10	24	8	14	14	3	4	1

*More than 1 microorganism involved.

(2 cases) and aspergillus (1 case) were found in 3 other patients with acute leukaemia. Oesophagitis and pyelitis caused by candida were found respectively in 1 and 2 patients, but were considered to have played no significant role in the fatal outcome of these patients. Fatal bilateral pneumonia associated with cultural and serological evidence of infection caused by adenoviruses or cytomegaloviruses was found in 2 patients. In the latter cases disseminated infection caused by cytomegaloviruses was also observed. Cutaneous infection with herpes zoster, disseminated schistozomiasis and pulmonary tuberculosis were also found each in one patient but were not considered to have played a significant role in the fatal outcome in these patients.

Haemorrhage

There were 18 (11·4%) episodes of severe haemorrhage that were considered to be directly responsible for death in this series, moreover, in 8 instances haemorrhage was a significant associated cause of death. Gastro-intestinal haemorrhage was the most frequent complication (14 cases) and originated from duodenal ulcers in 2 cases. In all other patients massive and diffuse intestinal haemorrhage were observed. Cerebral haemorrhage (meningeal or subdural) was found in 9 instances. Ruptured carotid artery and massive haemoptysis were responsible for 3 serious haemorrhages, one of which was fatal. Intraperitoneal haemorrhage was observed in association with gastro-intestinal bleeding in 1 case and massive haemopericardium was found to be associated with meningeal bleeding in another case.

Other causes

A significant number of patients in this series died as a direct consequence of neoplastic infiltration of vital organs (brain, lungs, liver) as shown in Table 2. Intractable hypercalcemia developed in 4 patients with extensive bone metastases. This relatively high incidence of cancer as a direct cause of death in this series was probably related to the far-advanced cancer present in most patients studied here.

Clinical correlations

Factors such as age, sex, duration of the malignant disease (more or less than 1 year), and the length of the last hospital stay (less than 1 week, less or more than 3 weeks) did not significantly influence the cause of death in the patients studied here. The role of the type of cancer in the causes of death is illustrated in Table 4. Only cases where a single cause was found to explain death were considered for this analysis. It can be seen that infection played a major role as a cause of death in patients with leukaemia, upper respiratory and digestive tract neoplasms and in those with gastro-intestinal and genito-urinary tract tumours. Only in patients with leukaemia and to a lesser extent in those with lymphoma did haemorrhage play a significant role as a cause of death. The frequency of the cancer itself being the cause of death in patients with lung or breast cancer was striking.

The type of antineoplastic therapy might also influence the cause of death in patients with cancer and the therapeutic factors were also analysed with respect to the relative incidence of the different causes of death considered in this study. As shown in Table 5, no clear-cut influence of the type of treatment on the cause of death could be found in this study, although infection seemed to be a more common cause of death in patients treated with cytostatic agents or radical surgery.

Infection was approximately twice as common as a cause of death in leukopaenic patients (less than 1500 granulocytes/mm^3) as in non-leukopaenic patients. The frequence of infection in leukopaenic patients presumably explaines the higher incidence of fatal infections in patients with leukaemia since all these patients were severely leukopaenic. However, factors other than granulocytopaenia [5] are of importance to predispose patients with cancer to severe infection, since the patients with intestinal, urinary or upper respiratory neoplasms had an incidence of fatal infections similar to that of leukemic patients. As might be expected, thrombocytopenia (less than 50,000 platelets/mm^2) was a significant factor in predisposing the patients to serious haemorrhage. Severe thrombocytopaenia was found in this series almost exclusively in patients with acute leukaemia. However, it should be pointed out that the mean level of platelets in patients with acute leukaemia was 20,500 platelets/mm^3 and that only 1 out of 21 patients with acute leukaemia had more than 100,000 platelets/mm^3 at the time of death. In spite of this severe degree of thrombocytopaenia, haemorrhage was the cause of death in 7 (33%) of these patients only. On the other hand the mean level of platelets in 18 patients who died from haemorrhage was 81,700/mm^3. Therefore, it might be suspected that causes other than the platelet level are involved in the pathogenesis of serious haemorrhage in patients with malignant lesions.

Table 4. Relationship between the type of cancer and the cause of death in patients in whom a single cause of death could be demonstrated

	Leukaemia		Lymphoma		Lung		Breast		Urinary and Genital		Upper respiratory and digestive	
	(23)		(19)		(19)		(17)		(19)		(17)	
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%
Infection	13	56	5	25	3	16	4	24	10	53	9	54
Haemorrhage	8	34	5	25	1	5	1	6	1	5	1	6
Cancer	1	5	4	25	12	63	9	54	4	21	4	23
Other	1	5	5	25	3	16	3	17	4	21	3	17

Table 5. Relationship between the type of anti-neoplastic therapy and the cause of death in patients in whom a single cause of death could be demonstrated

	No therapy No leukopaenia No thrombocytpaenia		Chemotherapy		Surgery		Radiotherapy		Leukopaenia		Thrombopaenia	
	(46)		(51)		(14)		(17)		(31)		(34)	
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%
Infection	16	34	21	41	8	56	5	29	19	61	15	44
Haemorrhage	8	17	6	11	1	6	3	18	6	19	12	35
Cancer	16	34	12	24	2	14	6	35	2	6	3	9
Other	6	15	12	24	3	24	3	18	4	14	4	12

It was not possible to study the effect on the causes of death of therapy with corticoids here since only 8 patients received corticoids in full dosage during their last stay in the hospital. Tests of delayed skin sensitivity (tuberculin, candidin, toxoplasmin) were available for most patients studied in this series. Determination of serum levels of immunoglobulins (IgA, IgG, IgM) were also available in most of these patients. No correlation between these factors and the causes of death or the type of infection could be found.

DISCUSSION

Only overwhelming fatal events were taken into consideration for this analysis and it should be emphasized that the incidence of non-fatal complications, which are usually associated with significant morbidity in patients with cancer, is not reflected here.

Studies of the causes of death in cancer may vary greatly [1, 6]. Obviously results may be influenced by the use of new antineoplastic drugs and depend greatly on the type of supportive therapy used in these patients who are the subject of the analysis of the causes of death. Moreover, results are also influenced by the thoroughness of the clinical bacteriological and pathological search for the cause of death in these patients. Therefore, our conclusions may be valid only for our hospital.

As for patients with acute leukaemia [1], infection was the leading cause of death in patients with various types of cancer. However, patients with lung and breast cancer die more often from neoplastic invasion of vital organs than from infection or haemorrhage.

Leukopaenia played an important role in predisposing to infection, especially in patients with acute leukaemia as might be expected [5]. However, patients with intestinal, genito-urinary and upper respiratory neoplasms died from infection as often as those with leukaemia in spite of normal levels of granulocytes in most of these patients.

One might speculate whether in these patients the colonization of sites of the body where resistance to infection is lowered by the presence of a neoplasm or therapy (surgery or radiation) has not played a major role in initiating potentially fatal infections. Colonization and superinfection of the respiratory tract

of patients treated for acute pneumonia, i.e. patients who presented a local diminution of the mechanisms of defence against infection is well documented [7].

The microorganisms responsible for infection in this series were predominantly gram-negative microorganisms. Most of them were highly resistant to commonly used antibiotics. The importance of resistant gram-negative rods in serious infections in hospitalized patients has been stressed several times in the past years [3, 8] and might explain, in part at least, why therapy of these infections is particularly difficult.

Haemorrhage was the cause of death in 10% of the patients in this series. It should be observed, however, that only 30% of patients with acute leukaemia died of haemorrhage in spite of severe thrombocytopaenia. Reduction of the incidence of fatal haemorrhage in leukaemic patients has been documented during the past years and is probably related to the use of platelet transfusions since platelets play a major role in the prevention of bleeding in these patients [9]. However, our data suggest that the level of platelets is not the only factor involved in the fatal haemorrhagic complications of patients with cancer.

One might question the overall significance of an analysis like the one we have performed; indeed only 10% of the patients studied were susceptible to a possible cure by modern treatment of cancer. However, present techniques of curing cancer may improve rapidly, as one can see for example from recent progress in the therapy of acute leukaemia, and better supportive care might allow more aggressive and more successful cytostatic, radiation and surgical treatment.

The data presented here show that bacterial infection is the major cause of death in cancer today, suggesting that a considerable effort should be made to prevent and more readily cure infections in these patients. Procedures intended to reduce the threat of infection in patients with cancer such as the adequate use of new antibiotics, alone or in combination, the transfusion of granulocytes and the realisation of a sterile environment should be carefully investigated and evaluated.

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Clinical Trial of 2-Br-α-Ergocryptine (CB154) in Advanced Breast Cancer*

EUROPEAN BREAST CANCER GROUP†

Abstract—2-Br-α-*ergocryptine was given orally for* 6 *weeks to* 19 *patients with advanced breast cancer. No objective remission was observed.*

2-Br-α-ERGOCRYPTINE (CB 154) is an ergot alkaloid having some pharmacological effects in the mouse and in the rat, that can be explained by its interference with the secretion of prolactin [1]. Furthermore, it inhibits prolactin secretion of the rat and human pituitary gland in organ culture [2]. In view of these data, CB 154 was tested on the DMBA-induced mammary carcinoma of the rat, which is prolactin-dependent [3]. It was found to inhibit growth of this tumour [4].

The purpose of this trial was to assess the therapeutic activity of the drug in advanced breast cancer.

MATERIAL AND METHODS

The criteria used for selection of patients and evaluation of results are those described in the protocol of the clinical trials of the Group [5], with minor modifications.

The main features of the protocol are biopsy proof of the disease, post-menopausal status of the patients, use of objective criteria of remission, evaluation of the results by two non-participating observers.

CB 154 (Sandoz, S.A., Basel, Switzerland) was administered at the following dosages for 6 weeks:

3 × 2 mg/day for the first 2 days,
3 × 3 mg/day for the next 2 days,
3 × 4 mg/day for the next 2 days,
3 × 5 mg/day for the rest of the 6 weeks.

RESULTS AND CONCLUSION

Nineteen patients were treated with CB 154. Table 1 shows the distribution of these patients according to the predominant lesion and the post-menopausal status. No objective remission was observed (0%; 95% confidence limit: 0–18%).

Table 1. *Distribution of patients and results in advanced breast cancer trial with CB 154*

Dominant lesion	Years after menopause				
	<1	1–5	5–10	>10	Total
Soft tissue	—	0/1	0/1	0/6	0/8
Osseous	—	0/1	—	0/2	0/3
Visceral	0/1	0/1	0/2	0/4	0/8
Total	0/1	0/3	0/3	0/12	0/19

The figures in the table give the number of objective remissions over the number of cases treated.

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†This Group is one of the Co-operative Groups of the European Organization for Research on Treatment of Cancer (EORTC). Chairman: J. C. Heuson, Secretary: A. Coune, Statistician: G. Vamecq, Representative of the EORTC Council: H. J. Tagnon, Co-ordinator: M. Staquet.

Participants to this study are: *Academisch Ziekenhuis Sint-Rafaël, Leuven, Belgium* A. Drochmans, J. Wildiers; *Hôpital Necker, Paris, France* A. Netter, A. Gorins; *Institut Jules Bordet, Brussels, Belgium* A. Coune, J. C. Heuson, J. Michel, R. Philippon, M. Staquet, H. J. Tagnon; *Rotterdamsch Radio-Therapeutisch Instituut, Rotterdam, The Netherlands* H. van Gilse, D. Blonk-van der Wijst.

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Clinical Trial of the Cyclic Imide 1-(Morpholinomethyl)-4-Phtalimido-Piperidindione-2,6 (CG 603) in Advanced Breast Cancer*

EUROPEAN BREAST CANCER GROUP†

Abstract—*An EORTC type* 1 *clinical trial of the cyclic imide CG* 603 *was conducted in patients with advanced breast cancer, according to the protocol of the European Breast Cancer Group (EORTC). One objective remission out of* 23 *cases evaluated was observed. The side-effects were nausea and vomiting, often transient, occurring in about half the patients. It is concluded that the drug possibly has a weak inhibitory effect on breast cancer.*

INTRODUCTION

CG 603 (Chemie Grünenthal, Stolberg, D.B.R.) is a thalidomide derivative found to be effective in producing tumour regression of DMBA-induced rat mammary carcinoma [1–7]. The mechanism of action might be due to inhibition of prolactin secretion [8–10].

The purpose of this trial was to assess the therapeutic activity of the drug in advanced breast cancer.

MATERIAL AND METHODS

The criteria used for selection of patients and evaluation of results are those described in the protocol of the clinical trials of the Group [11], with minor modifications.

The main features of the protocol are biopsy proof of the disease, post-menopausal status of the patients, use of objective criteria of remission, evaluation of the results by two non-participating observers. The dose was 2 g/day orally, divided into 4 doses, given over a period of 6 weeks.

RESULTS

Table 1 gives the number of patients, their distribution in the different categories and the number of objective remissions in a total of 23 patients treated for at least 14 days. There was only one objective remission (4·3%; 95% confidence limit: 0·1–22·0%).

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Participants to this study are: *Academisch Ziekenhuis Sint-Rafaël, Leuven, Belgium* A. Drochmans, J. Wildiers. *Antoni van Leeuwenhoek-Huis, Amsterdam, The Netherlands* E. Engelsman. *Hôpital Necker, Paris, France* A. Netter, A. Gorins. *Institut Jules Bordet, Brussels, Belgium* A. Coune, J. C. Heuson, J. Michel, R. Philippon, M. Staquet, H. J. Tagnon. *Istituto Regina Elena, Rome, Italy* G. Jacobelli. *Kantonsspital Zurich, Zurich, Switzerland* G. Martz, H. Favre. *Universitäts-Krankenhaus Eppendorf, Hamburg, D.B.R.* H. Nowakowski.

Table 1. *Clinical trial with* CG 603 *in advanced breast cancer. Distribution of patients and results*

Dominant lesion	Years after menopause				Total
	1	1–5	5–10	10	
Soft tissue	0/2	—	0/1	0/2	0/5
Osseous	—	0/4	0/1	0/3	0/8
Visceral	0/2	0/3	1/2	0/3	1/10
Total	0/4	0/7	1/4	0/8	1/23

The figures in the table give the number of objective remissions over the number of cases treated.

The only definite side-effect recorded was nausea (13 cases), which was sometimes associated with vomiting (5 cases). In the majority of cases, these symptoms subsided despite continuation of therapy. However, they were severe enough in 3 cases to necessitate premature arrest of the treatment. The treatment was interrupted in 6 other cases because there was evidence of rapid progression before completion of the 6-week period of therapy.

There was no sign of haematological, renal or hepatic toxicity.

DISCUSSION

This clinical trial suggests that CG 603 administered at a daily dose of 4×500 mg by the oral route is hardly active in the treatment of the advanced phase of breast cancer. In fact, the probability that the drug is active in 20% or more of the parent population is at most 0·04. The weakness of this effect, as expressed by the low rate and poor quality of remission, could possibly be due to an insufficient intestinal absorption of the drug.

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The Effect of a Complex Yeast Preparation, as a Food Supplement, on the Growth of Ehrlich's Ascites Tumour in Mice

J. D. IRESON, G. E. CONWAY and F. H. SCHWARZENBACH
Applied Biology Department, North East London Polytechnic, Longbridge Road, Dagenham, Essex, England

Abstract—*The effect of a complex yeast preparation, as a food supplement, has been investigated on the growth of Ehrlich's ascites tumour in ascitic and solid form in mice. Animals were premedicated with the preparation for* 14 *days before cancer inoculation, and medication then continued to completion of the experiment. Tumour growth was reduced in all groups treated with the preparation, as compared to control animals treated with saline. The preparation was more effective in male animals than in female animals. The preparation appears to antagonise the establishment ("take") and initial growth of the cancer. Its possible mechanism of action is discussed.*

INTRODUCTION

CLINICAL and statistical findings suggest that the genesis of some kinds of tumours in man is partly dependent on the nutritional state, that is, whether the individual is overweight or underweight. The results of several surveys [1] imply that individuals who overeat and are overweight when past middle-age are more likely to die of cancer than persons of average weight or less.

Among the earliest studies on nutrition in relation to cancer are those indicating that underfeeding retards the growth of transplanted tumours [2–4]. Later this was shown to be a consequence of restriction in calories; i.e. it could be produced by reduction of any one component of the diet, for instance, the carbohydrate (or fat) content of the diet [5].

As with restriction of calories, protein deprivation decreases the rate at which neoplasms become established and grow [6, 7].

There is no evidence that the formation or growth of neoplasms is influenced by altering the proportions of inorganic components natural to the diet. The literature contains many contradictory reports; and the best studies, in the main, conclude that there are no important effects [8, 9].

Numerous attempts have been made to link carcinogenesis with both deficiencies and abundances of vitamins. However, vitamin deprivations usually result in lower food intake and body weight, and a shortened life span, events which in themselves either modify the onset of neoplasms, or do not permit the completion of the process. The majority of the vitamins appear to have no noteworthy effects [9–11]. It has been reported that relative deficiencies of riboflavin, biotin and pyridoxine hinder the genesis and development of neoplasms [12–14]. However, a survey of 34 diets made by Miller *et al.* [15] demonstrated that dietary supplements rich in both protein and B vitamins, particularly riboflavin, inhibited tumour formation. Similar findings have been reported by Sugiura [16, 17], who fed rats for a limited period on *p*-dimethylaminoazobenzene (butter yellow) in a brown rice diet, to induce liver tumours, and then transferred the animals onto dye-free rations of

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brown rice, brown rice supplemented with yeast, or brown rice supplemented with milk powder. The incidence of liver neoplasms were significantly lower in the rats given the yeast or milk powder.

We therefore decided to investigate the influence of a dietary supplement, a complex yeast preparation, on the rate and degree of growth of Ehrlich's tumour in mice.

METHOD

The tumour used was Ehrlich's ascites, and two types of experiments were performed, one where the tumour was administered intraperitoneally, when it remained ascitic [18], and the other when it was administered subcutaneously, when it became solid [19].

The complex yeast preparation used was a suspension of plasmolyzed wild yeast (Candida utilis) and known to contain a wide variety of natural vitamins, minerals, amino-acids, and active enzymes (Tables 1–3).

In all experiments the animals were allowed normal food (Dixon's diet No. GR3EK) *ad libitum*.

Ascitic form

One hundred and sixty Schofield Swiss mice, weight range 18 to 22 g, were divided into two groups, one comprising 80 female animals, the other 80 male animals. The 80 female animals were sub-divided into two groups of 40 animals, one of which was dosed with 0·1 ml saline daily, the other with 0·1 ml of the yeast preparation daily. After 14 days, all the animals were inoculated with 30,000 ascites cancer cells in a 0·2 sample, each. Daily dosing with the yeast preparation or saline was continued until the animals were examined.

Seven days after inoculation, 20 animals from each group were killed, and the ascitic fluid withdrawn. The volume of the ascitic fluid, and its cellular contents were examined for each mouse. Fourteen days after inoculation the remaining 20 animals in each group were killed and examined in the same way. The 80 male animals were treated identically.

Table 1. *The physico-chemical properties of the yeast preparation*

	Unit	
Specific weight		1·283
pH (electrometric)		5·06
Energy content	cal/100 ml	346·00
Water	vol %	35·50
Acid (total acid)	g/100 ml	0·60
Protein ($f = 6·25$)	g/100 ml	7·10
Total fats	g/100 ml	0·96
Minerals	g/100 ml	0·80

Table 2. *The vitamin and mineral content of the yeast preparation*

Vitamins		Unit	
B 1	thiamine	mg/100 g	0·30
B 2	riboflavin		0·77
B 6	pyridoxine		0·18
	nicotinamide		3·73
H	biotin		0·0032
	pantothenic acid		6·90
	folic acid		0·54
B 12	cobalamin		0·00021
C	ascorbic acid		3·21
	inositol		67·50

Minerals		Unit	
Aluminium	(Al)	mg/100 g	0·92
Lead	(Pb)		0·0306
Calcium	(Ca)		8·80
Chlorine	(Cl)		79·04
Iron	(Fe)		3·44
Potassium	(K)		217·50
Silicic acid	(Sl)		6·45
Cobalt	(Co)		0·04
Copper	(Cu)		0·24
Magnesium	(Mg)		17·07
Manganese	(Mn)		0·0936
Sodium	(Na)		4·75
Phosphorus	(P205)		300·00
Total sulphur	(S)		28·09

Table 3. *The amino-acid content of the yeast preparation*

Amino acids	Unit	
Arginine	mg/100 g	350
Cystine		63
Glutamic acid		780
Glycine (glycocoll)		215
Histidine		120
Isoleucine		480
Leucine		260
Lysine		490
Methionine		45
Phenylalanine		210
Threonine		170
Tryptophan		53
Tyrosine		50
Valine		260
Choline		60
Glutathione		32

Solid form

Two hundred and forty Schofield Swiss mice, weight range 18 to 22 g, were divided into two groups, one comprising 120 female animals, the other 120 male animals. One hundred and twenty female animals were further subdivided into two groups of 60 animals, one of which was dosed with 0·1 ml saline daily, the other with 0·1 ml of the yeast preparation daily. After 14 days, all the animals were inoculated in the region of the groin with the cancer, each mouse receiving a 0·2 ml inoculum containing 30,000 ascites cells. Daily dosing with the yeast preparation or saline was continued until the animals were examined.

Fourteen days after inoculation, 20 animals from each group were killed, and the solid tumour dissected free. Each individual tumour was then weighed and examined for degeneration. Twenty animals from each group were examined in the same fashion 21 and 28 days after inoculation. The 120 male animals were treated identically.

Sterility of the yeast preparation

Prior to and following the experiments, the yeast preparation was tested for sterility by streaking it onto a nutrient agar plate. No growth was observed after 72 hr incubation at 37°C; the sample was thus considered to be sterile.

RESULTS

Ascitic form

The mean volume of the ascitic fluid in animals treated with the yeast preparation was significantly less than the volume of ascitic fluid in animals treated with saline (Table 4).

A better measurement of cancer growth is that of total ascitic cancer cells per mouse, i.e. volume of ascitic fluid times number of cancer cells per ml. Accordingly, each individual sample was assayed for the number of cancer cells it contained, and as can be seen from the Table 5, there was a significant reduction in the

Table 4. Average amount of ascitic fluid per mouse in control animals and animals treated with the yeast preparation, and a statistical comparison of the two groups using the Students' "t" test

No. days following cancer inoc.		Average amount of ascitic fluid per mouse peritoneum (ml)			
		Controls	Treated with yeast preparation	"t"	"p"
7	Female mice	9·0	5·6	3·2	<0·001
	Male mice	10·6	6·9	3·3	<0·001
14	Female mice	14·6	11·0	2·0	<0·05
	Male mice	13·9	7·7	5·8	<0·001

Table 5. The mean ascitic cancer cells per peritoneum (millions) in control animals and animals treated with the yeast preparation, and the statistical comparison

No. days following cancer inoc.		Mean number cancer cells per peritoneum (millions)			
		Controls	Treated with yeast preparation	"t"	"p"
7	Female mice	2·4	1·2	4·23	<0·001
	Male mice	3·1	1·4	4·04	<0·001
14	Female mice	8·0	5·4	2·22	<0·05
	Male mice	8·7	4·6	3·52	<0·001

mean number of cancer cells per mouse, in those animals treated with the yeast preparation, as compared to those dosed with saline.

It was also noticeable that the incidence of haemorrhagic tumours, caused by advanced tumour growth damaging peritoneal blood vessels and leading to blood leakage into the peritoneal cavity, was reduced in the animals treated with the yeast preparation. An incidence of 15 to 20% after 7 days was reduced to 5% and an incidence of 40 to 45% after 14 days was reduced to 25%.

Solid form

In the test situation where the cancer was inoculated subcutaneously to the region of the groin, the weight of the cancers removed from the mice treated with the yeast preparation was less than the weight of the cancers of the untreated animals (Table 6). There was a notable reduction in the number of cancers which had degenerated, became necrotic, in the yeast preparation treated groups as compared to the controls. An incidence of 5 to 10% after 21 days was reduced to 0%, and an incidence of 25 to 30% after 28 days was reduced to 10–15%.

Table 6. The average weight of tumour per mouse (g) in control animals treated with the yeast preparation, and the statistical evaluation

No. days following cancer inoc.		Average weight of tumour per mouse (g)			
		Controls	Treated with yeast preparation	"t"	"p"
14	Female mice	1·00	0·77	1·5	$>0{\cdot}1$
	Male mice	1·07	0·79	1·8	$0{\cdot}1<P<0{\cdot}05$
21	Female mice	1·46	1·22	1·6	$>0{\cdot}1$
	Male mice	1·43	1·30	1·1	$>0{\cdot}1$
28	Female mice	2·24	2·03	0·7	$>0{\cdot}1$
	Male mice	2·24	2·03	1·0	$>0{\cdot}1$

Statistical evaluation of experiments with the ascitic form of the tumours

General experience has shown that in the initial phase, processes of growth and propagation of the cancer consist of a lag phase and then follow an exponential function. Because of this, we have calculated the logarithms of the original figures for ascitic volume and cancer cells/ml. The simplest way of graphically representing the results of the experiments is to plot the figures as a correlation in a rectangular two-dimensional system of coordinates, the y axis being log c (cancer cells/ml), the x axis being log v (ascitic fluid volume), as in Fig. 1. Such analyses demonstrate that the data for treated and untreated mice fall in different groups. Individual values for animals given the yeast preparation are generally showing lower ascitic fluid volumes and lower cell concentrations. The logarithms of the original data were then used to obtain the means and the standard deviations for the logarithms of both volumes and cell concentrations. Our first evaluation concerned the means in comparative groups, assessed by using the t-test.

Table 7 gives the results of comparing the two mean values for each pair of groups.

Comparison of the mean values shows that differences were more evident statistically in the groups of male mice than in the groups of female mice.

The mean values for log v and log c in all four groups fed on the yeast preparation were below those of the comparable control groups, though the degree of significance varied. In five out of the eight comparisons, using the t-test, significance, being at the level of $P = 0{\cdot}05$, was established.

From these comparisons we draw the conclusion that the yeast preparation has an inhibiting effect on tumour development. But so far the evaluation does not indicate if this inhibition may be regarded as due to a slowing down of tumour growth, to a delay in the beginning of growth, or to limits being set to growth.

In order to get some pointers in this important respect, we made an assessment of the mean

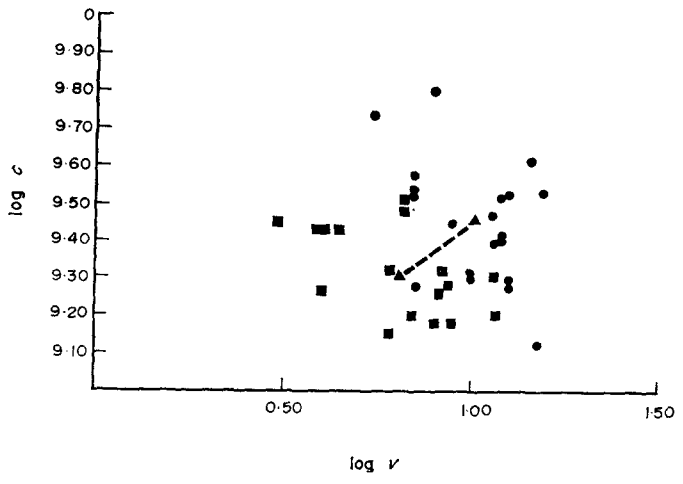

Fig. 1. A rectangular two-dimensional plot of the experimental results obtained from male animals examined 7 days after cancer inoculation. Log c *is the logarithm of the cancer cells/ml ascitic fluid; log* v *is the logarithm of the ascitic fluid volume.*

It can be seen that the control (●) and treated (■) groups are falling in different ellipses within the area of the graph, indicating that the degree of growth of the cancer in the treated animals is less than in the controls. The means (▲——▲) of the two groups are also indicated.

Table 7. "t" *tests to compare the mean log* c *(cancer cells/ml) and the mean log* v *(ascitic volume) in control animals and animals treated with the yeast preparation*

	Mean log c			Mean log v		
Groups compared	Difference in mean values	"t"	P	Difference in mean values	"t"	P
Male 7 days control/treated	−0·14	3·02	<0·01	−0·20	4·08	P_{35}<0·001
Female 7 days control/treated	−0·12	2·49	<0·02	−0·12	1·93	P_{34}<0·10
Male 14 days control/treated	−0·12	2·07	<0·05	−0·21	4·39	<0·001
Female 14 days control/treated	−0·06	0·67	<0·50	−0·10	1·97	<0·10

values in comparable groups after 7 and 14 days. From the results we found that log v increases much more with the females than with the male animals. The increase in the volume of ascitic fluid therefore shows a dependence on sex.

The increase in cell concentration per ml, on the other hand, is of comparable extent in male and female animals.

These findings are clearly apparent in the graphical representation in Fig. 2.

The sex-dependent changes can be shown up by calculating the quotient $Q = v/c$ ("change in volume from 7th to 14th day" divided by "change in cell concentration from 7th to 14th day"). The following quotients were calculated for the different groups:

male, control, $Q = \frac{0{\cdot}33}{0{\cdot}11} = 3{\cdot}0$;

male, treated with the yeast preparation, $Q = \frac{0{\cdot}35}{0{\cdot}10} = 3{\cdot}5$;

female, control, $Q = \frac{0{\cdot}26}{0{\cdot}31} = 0{\cdot}9$;

female, treated with the yeast preparation, $Q = \frac{0{\cdot}32}{0{\cdot}24} = 1{\cdot}3$.

These quotients, which must be seen as tangential to the angle of inclination of the straight line connecting the points in Fig. 2 show marked deviation between the two sexes.

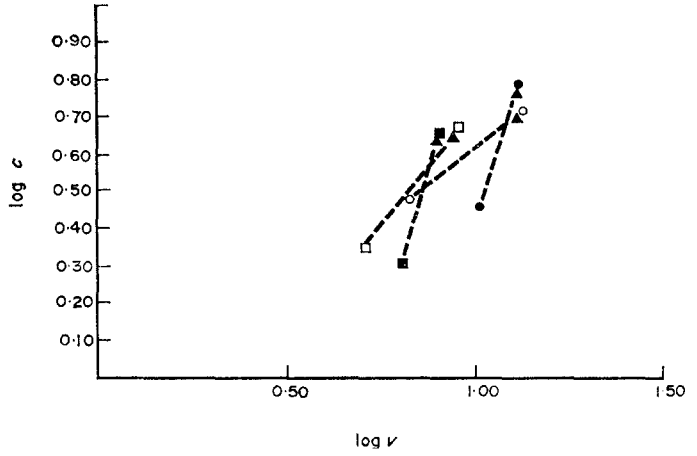

Fig. 2. *A rectangular two-dimensional plot comparing the mean values log* v (*ascitic fluid volume*) *and log* c (*cancer cells/ml ascitic fluid*) 7 *and* 14 *days after cancer inoculation.*

The change in the relation of cancer cells to ascitic fluid volume over the 7 *day period can be seen for the control male* (●) *and female* (○) *animals and the treated male* (■) *and female* (□) *animals.*

Statistical evaluation of experiments in which the solid form of the tumour was used

Preliminary statistical calculations have given some indication that during the experimental period the tumours are in the phase of exponential growth. During this stage of development the rate of increase is proportional to the existing mass of the tumour.

When this became obvious, we took the logarithms of the weights and used these for statistical calculations. The mean values for the twelve different groups are given in Table 8.

The following may be concluded from this table:

(1) All the animals treated with the yeast preparation showed lower tumour weights than the controls. In the first two groups compared, the differences are statistically significant ($P = 0{\cdot}05$).

(2) Unlike with ascites tumours, no difference was found between male and female animals. Because of this, the experimental groups male and female may be taken together.

For the further assessment of results we have used graphical representation, with the mean log W (tumour weight) combining male and female groups (Fig. 3).

The following conclusions may be drawn from the graph:

(1) The points lie on straight lines. This confirms what was suspected before, that during the experiment tumour growth was in the exponential phase.

(2) The two lines differ in their position which indicates that the tumour growth starts later in animals treated with the yeast preparation; the stage when the weight of the tumour is 1·00 g (log $W = 0{\cdot}00$) is passed about 3 days

Table 8. *Comparison of mean values log* W (*tumour weight*) *of Ehrlich's tumour in control animals and animals treated with the yeast preparation*

Group		Control		Treated			
		No	log W	No	log W	"t"	P
14 days	Male	20	9·98	20	9·84	2·00	0·05
	Female	17	9·96	20	9·85	2·220	<0·05
21 days	Male	20	0·14	20	0·10	1·00	<0·40
	Female	20	0·13	20	0·06	1·36	<0·20
28 days	Male	20	0·35	20	0·28	1·72	<0·10
	Female	17	0·32	20	0·28	0·75	<0·50

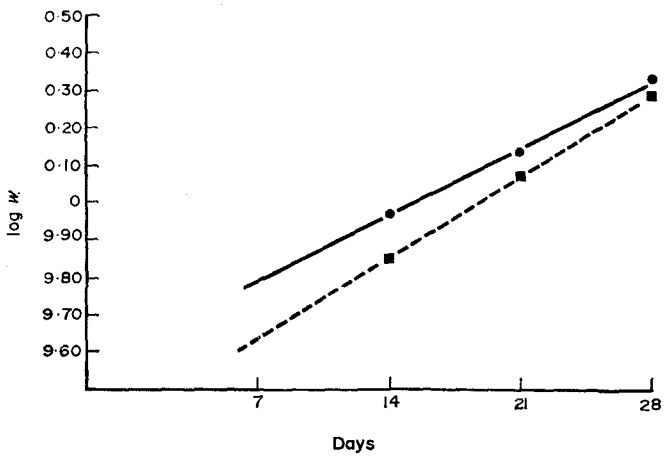

Fig. 3. The rate of growth of the Ehrlich's ascites, solid form tumour in control (●) and treated (■) animals over the period 14–28 days following cancer inoculation. Log W is the mean of the logarithms of the individual tumour weights.

later than in the controls. Treatment with the yeast preparation therefore delays the beginning of tumour growth.

DISCUSSION

It can be seen from the results that in both the ascitic and solid form experiments, the yeast preparation reduced the degree of growth of the cancer. In the ascitic form experiments, the ascitic volume, total cancer cells, and incidence of haemorrhage were all reduced, and in the solid form experiments, the cancer size, and the incidence of necrosis were both reduced.

Food intake of the pellets normally provided was not significantly diminished in any group. The effectiveness of the yeast preparation was not, therefore, that due to its influence, the animals were reducing their normal intake of food, and suffering a specific dietary deficiency.

It can be seen by comparing Figs. 1 and 3 that the division between the control and treated animals at day 7 is more marked than at day 14. This, together with the results from the solid form experiments (Fig. 3), which show that the cancer development appears delayed in these animals treated with the yeast preparation, indicates that the effect of pre-treatment with the yeast preparation is to antagonise the "take" or early growth of the cancer.

It would appear from these results that probably by use of a food supplement we are providing a factor which might be lacking from the animal's normal diet, and which contributes to the general level of health of the animal, its natural resistance, and thereby antagonises the establishment of the cancer.

From the results of the ascitic form experiments, that male animals respond more effectively to the treatment than female animals, it is conceivable that the latter are lacking in some metabolic function necessary to utilise the factor provided by the yeast preparation.

The data presented does not rule out a direct effect of the preparation on the tumour, unconnected with any dietary requirement. It could be more effective early on because there are fewer cells to attack.

However, since the preparation appears to act by antagonising the "take" of the cancer, in subsequent experiments we intend to investigate its effectiveness against cancer dissemination and the development of metastases.

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Specific Growth Regulation in Three Ascitic Tumours*

P. BICHEL

Cancer Research Institute, Danish Cancer Society, Radiumstationen, DK 8000 Aarhus C, Denmark

Abstract—*Simultaneous inoculation of two different hypotetraploid ascites tumours in the same mouse was followed by independent development of both tumours. The total number of tumour cells reached a maximum of about 2×10^9, i.e. twice the number of cells found in the plateau phase of growth of a single ascites tumour.*

Injection of cell-free ascites obtained from one ascites tumour in the plateau phase of growth into mice bearing a different kind of ascites tumour was not followed by inhibition of the tumour cells in the G-1 and the G-2 phase of the cell cycle, which is otherwise seen when the ascites is injected into mice bearing ascites tumours of the same origin.

It is suggested that these findings might be explained by a tissue specific growth regulation similar to the chalone regulation of some normal tissues.

INTRODUCTION

PREVIOUS experiments have demonstrated that the growth of the JB–1 ascites tumour in mice is apparently regulated by a humoral negative feedback mechanism. An inhibitor of the cell cycle of the JB–1 ascites tumour cells was demonstrable in the acellular ascites fluid from JB–1 ascites tumour in the plateau phase of growth. Following injection of cell-free ascites fluid tumour cells were arrested in both the G–1 and in the G–2 phase [1].

The aim of the present experiments was to investigate the possible specificity of this reaction. Recently it has been demonstrated that simultaneous inoculation of two different ascites tumours in the same host is followed by almost complete additive growth [2]. Besides demonstrating that nutritional deficiency and metabolic waste apparently play only minor roles, the independent development of both ascites tumours rather indicates that the decreasing growth rate is due to a specific growth inhibition.

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†Kindly supplied by Dr. J. Kieler, the Fibiger Laboratory, Copenhagen.

MATERIALS AND METHODS

Mixed ascites tumours

Two ascites tumours, the JB–1 and a hypotetraploid line of the Ehrlich ascites tumour†, were inoculated intraperitoneally in our inbred BALB/c strain of mice. The mice were inoculated with $2{\cdot}5 \times 10^6$ JB–1 + $2{\cdot}5 \times 10^6$ Ehrlich ascites tumour cells. The controls received either $2{\cdot}5 \times 10^6$ JB–1 or $2{\cdot}5 \times 10^6$ Ehrlich ascites tumour cells. Four, 10, 12 and 14 days after inoculation, the mice were killed by cervical dislocation and the total number of tumour cells was determined according to the method described by Klein and Révész [3].

Chromosome analysis

The chromosome preparations were made according to the following instructions, which is a modification of the method described by Tjio and Whang [4]:

(1) Centrifugation of a small amount of the ascites tumour at 200 **g** for 8 min. (2) Removal of the supernatant. (3) Addition of 5 ml of 0·95% sodium citrate. (4) Incubation at 37°C for 15 min. (5) Addition of 6–9 drops of freshly prepared fixative consisting of 1 part of glacial acetic acid (Merck) and 3 parts of absolute methanol (Merck). (6) Centrifugation of the sample at

200 **g** for 8 min. (7) Removal of the supernatant and careful addition of new fixative, one drop at a time, with careful shaking of the sample until a total volume of ½ ml is obtained. (8) Addition of another 1–2 ml of fixative in larger portions. (9) Centrifugation of the sample at 200 **g** for 8 min. (10) Removal of the supernatant and new addition of 5 ml of fixative. (11) Incubation of the sample at −20°C for at least 1 hr. (12) Centrifugation at 200 **g** for 8 min. (13) Addition of freshly made fixative till opaque appearance of the cell suspension. (14) Three to 4 drops of the cell suspension are placed on cleaned slides cooled on dry ice, and maximum spreading of the cells is obtained by blowing vigorously alternating with drying in a flame. (15) The preparations are stained in 10% Giemsa in 0·05 molar phosphate buffer at pH 7·2. (16) The percentage of metacentric and submetacentric chromosomes is determined by counting at least 100 mitotic figures from each animal.

"Crossed treatment" with ascites fluid

In the second part of this investigation three different ascites tumours have been used. The JB–1 ascites tumour growing in our AK strain of mice, the Ehrlich ascites carcinoma growing in the C3H strain of mice, and HB, a mammary carcinoma which appeared spontaneously in our C3H mice in 1956. The HB tumour has been maintained by subcutaneous transplantation in the C3H strain, but was easily converted to ascites form in July 1970. The ascites tumours are maintained by intraperitoneal passage of 5×10^6 tumour cells every ninth or tenth day. The mice about two months old were kept on Althromin(R) and tap water *ad libitum*.

The mice were inoculated with $1{\cdot}0 \times 10^6$ ascites tumour cells. Ten days after inoculation as much as possible of the ascites tumour was aspirated leaving about 100 to 200×10^6 tumour cells, and 24 hr later the ascites tumours were investigated in the recurrent phase of growth [5].

Cell-free ascites fluid was obtained from donor mice inoculated with $2{\cdot}5 \times 10^6$ tumour cells. Eleven days later the ascites tumour was aspirated under sterile conditions. After centrifugation at 1500 **g** for 10 min the acellular ascites fluid was injected immediately.

Labelling index determined by means of autoradiography with H^3-thymidine and analysis of the accumulation of cells in mitosis after treatment with Colcemid(R) have previously been described [1].

RESULTS

Ten to 14 days after inoculation of either $2{\cdot}5 \times 10^6$ JB–1 or $2{\cdot}5 \times 10^6$ Ehrlich ascites tumour cells the total number of tumour cells reaches a plateau of about 10^9 (Fig. 1, Table 1). In accordance with the investigation by Brown [2] simultaneous inoculation of $2{\cdot}5 \times 10^6$ JB–1 and $2{\cdot}5 \times 10^6$ Ehrlich ascites tumour cells is found to follow an almost complete additive growth curve during the same period.

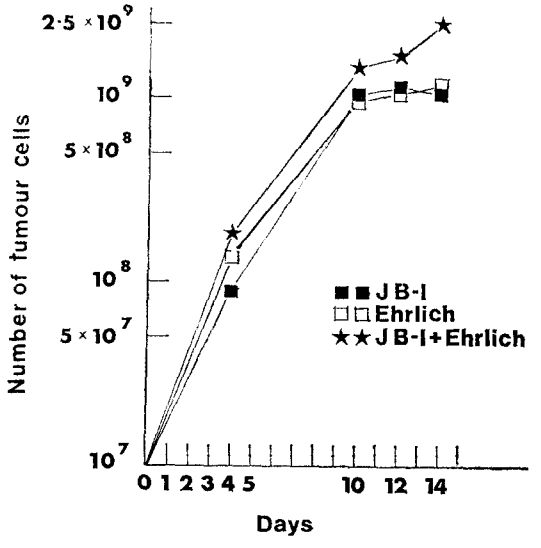

Fig. 1. Growth curve (semilogarithmic) of the JB–1 (■–■), the Ehrlich (□–□) and the mixed JB–1 + Ehrlich (★–★) ascites tumours.

Table 1. Total intraperitoneal number $\times 10^6$ *of tumour cells* $\pm$*S.D. following injection at day zero of* $2{\cdot}5 \times 10^6$ *JB–1 (■—■),* $2{\cdot}5 \times 10^6$ *Ehrlich (□—□) or* $2{\cdot}5 \times 10^6$ *JB–1* $+ 2{\cdot}5 \times 10^6$ *Ehrlich ascites tumour cells (★—★). In ( ) number of animals (see Fig. 1).*

Days after inoculation	4	10	12	14
■–■	88 ± 15 (6)	1058 ± 166 (6)	1158 ± 66 (7)	1062 ± 200 (4)
□–□	138 ± 40 (6)	950 ± 240 (4)	1055 ± 290 (7)	1200 ± 200 (7)
★–★	185 ± 17 (6)	1450 ± 200 (6)	1700 ± 370 (6)	2440 ± 300 (3)

S.D. = standard deviation.

In order to distinguish JB–1 from Ehrlich ascites tumour cells the chromosome picture of the tumours was investigated. To collect a sufficient number of mitotic figures the ascites tumour-bearing mice were treated with intraperitoneal Colcemid(R), 1 mg per kg for 3 hr. Figure 2 and Table 2 show the chromosome picture of JB–1 and Ehrlich cells demonstrating differences in the number of meta- and submetacentric chromosomes. Figure 3 and Table 2

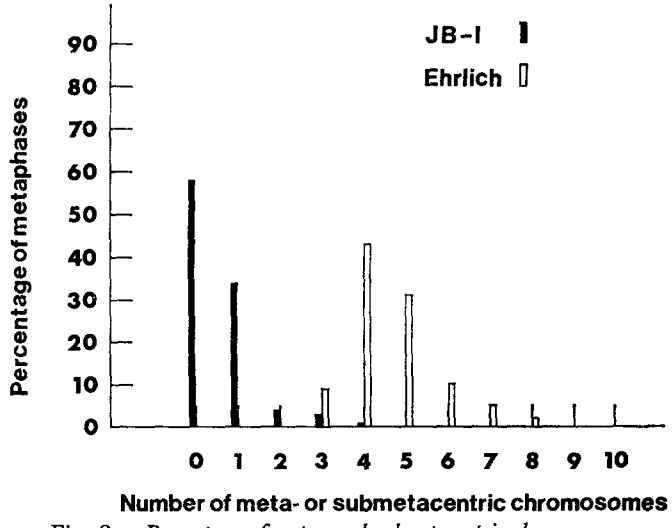

Fig. 2. Percentage of meta- and submetacentric chromosomes in the mitotic figures of the JB–1 and the Ehrlich ascites tumours.

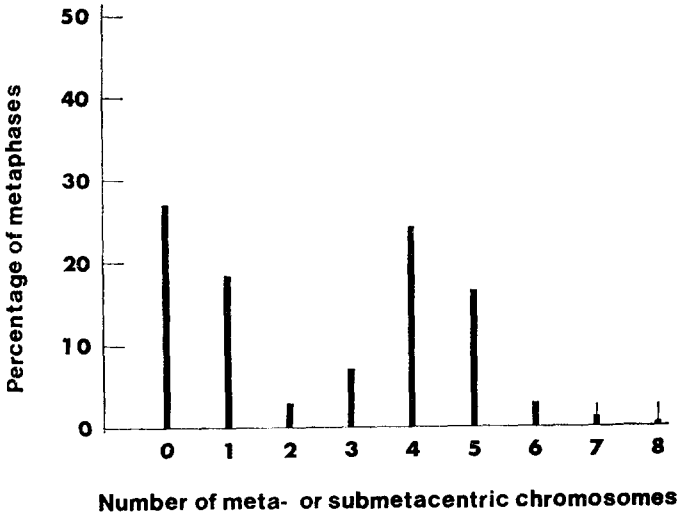

Fig. 3. Percentage of meta- and submetacentric chromosomes in the mitotic figures of the mixed JB–1 + Ehrlich ascites tumour.

Table 2. Percentage of meta- and submetacentric chromosomes ± S.D. in JB–1, Ehrlich, and JB–1 + Ehrlich ascites tumours. (See Figs. 2 and 3)

Ascites tumour	No. of animals	No. of meta- and submetacentric chromosomes ± S.D.								
		0	1	2	3	4	5	6	7	8
JB–1	2	58	34	4	3	1				
Ehrlich	2				9	43	31	10	5	2
JB–1 + Ehrlich	6	26·8 ± 1·9	18·3 ± 2·1	2·8 ± 0·6	7·0 ± 4·4	24·1 ± 2·6	16·5 ± 4·6	2·8 ± 1·0	1·1 ± 0·8	0·3

S.D. = standard deviation.

show the distribution of meta- and submetacentric chromosomes in mice with both JB–1 and Ehrlich ascites tumours.

Apparently, the increased number of tumour cells in the mixed ascites tumour is due to almost equal participation of the two tumours, although it has to be taken into account that the accumulation rate of the mitotic cells following treatment with Colcemid(R) is not necessarily the same.

Figures 4 and 5 illustrate the G–2 inhibitory effect of cell free Ehrlich ascites on JB–1 ascites tumours in recurrent growth and the effect of cell free JB–1 ascites on Ehrlich ascites tumours in recurrent growth (see legends and Tables 3 and 4).

Figures 6 and 7 show the G–2 inhibitory effect of cell free HB ascites on JB–1 ascites

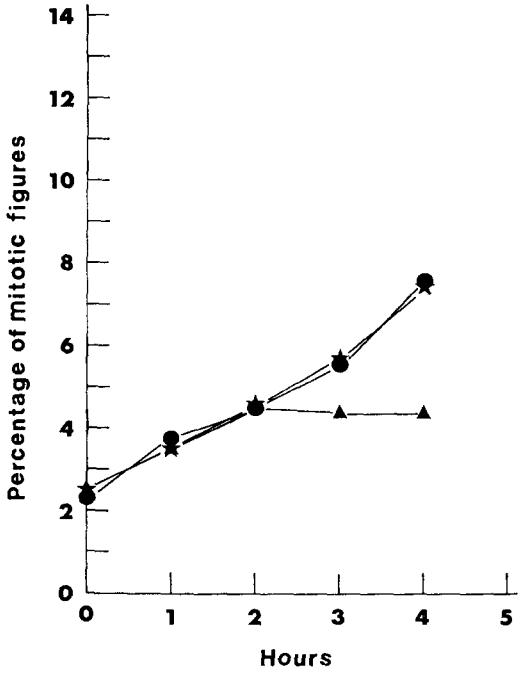

Fig. 4. Accumulation of recurrent growth JB–1 ascites tumour cells in mitosis determined with Colcemid(R) injected at zero hour. Controls (★–★). Addition of 2 ml of JB–1 ascites fluid at zero hour (▲–▲). Addition of 2 ml of Ehrlich ascites fluid at zero hour (●–●).

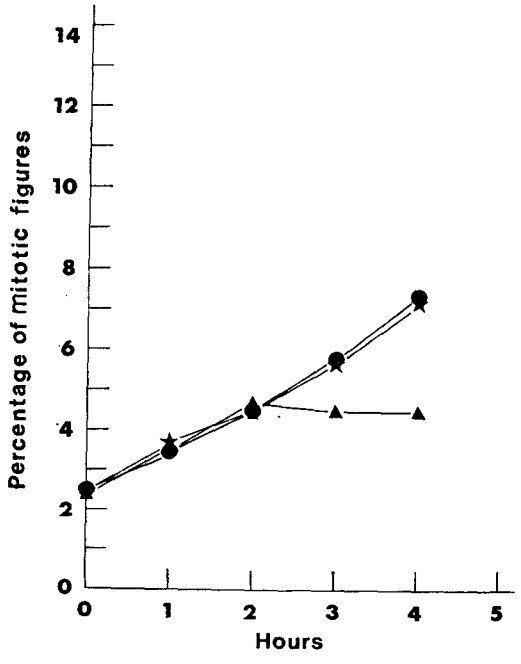

Fig. 5. Accumulation of recurrent growth Ehrlich ascites tumour cells in mitosis determined with Colcemid(R) injected at zero hour. Controls (★–★). Addition of 2 ml of Ehrlich ascites fluid at zero hour (▲–▲). Addition of 2 ml of JB–1 ascites fluid at zero hour (●–●).

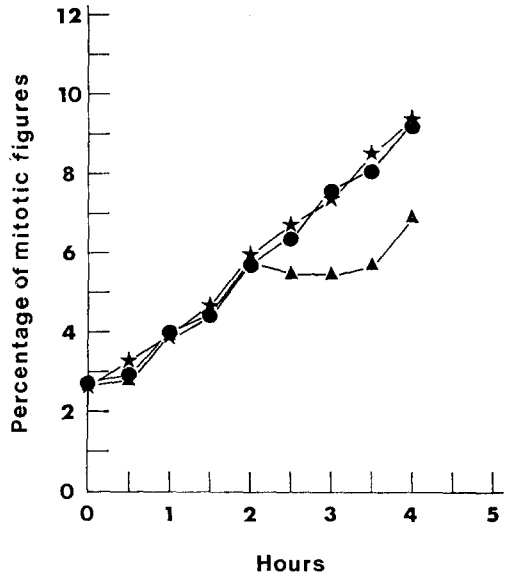

Fig. 6. Accumulation of recurrent growth JB–1 ascites tumour cells in mitosis determined with Colcemid(R) injected at zero hour. Controls (★–★). Addition of 2 ml of JB–1 ascites fluid at zero hour (▲–▲). Addition of 2 ml of HB ascites fluid at zero hour (●–●).

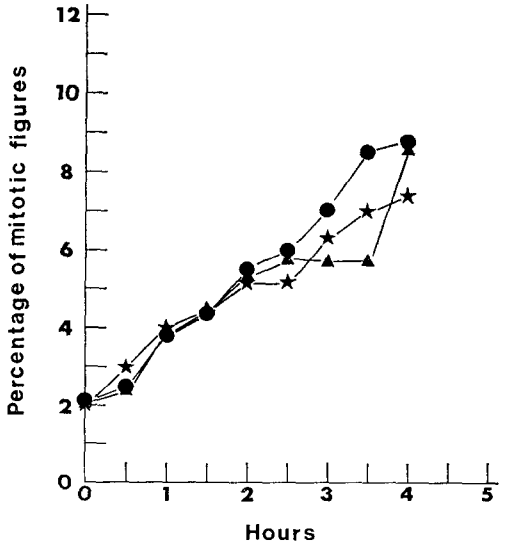

Fig. 7. Accumulation of recurrent growth HB ascites tumour cells in mitosis determined with Colcemid(R) injected at zero hour. Controls (★–★). Addition of 2 ml of HB ascites fluid at zero hour (▲–▲). Addition of 2 ml of JB–1 ascites fluid at zero hour (●–●).

Table 3. Percentage of mitotic figures in the JB–1 ascites tumour following injection of Colcemid(R) at time zero. Untreated controls (★—★). Injection of 2 ml of JB–1 ascitic fluid at zero hour (▲—▲). Injection of 2 ml of Ehrlich ascitic fluid at zero hour (●—●). (See Fig. 4)

	No. of animals	Mean value of percentage of mitotic figures ± S.D.				
		0 hr	1 hr	2 hr	3 hr	4 hr
★–★	6	2·5 ± 0·2	3·5 ± 0·2	4·6 ± 0·2	5·7 ± 0·1	7·4 ± 0·3
▲–▲	8	2·5 ± 0·2	3·5 ± 0·2	4·5 ± 0·2	4·4 ± 0·2	4·4 ± 0·3
●–●	8	2·3 ± 0·3	3·8 ± 0·2	4·5 ± 0·2	5·6 ± 0·3	7·5 ± 0·4

S.D. = standard deviation.

Table 4. Percentage of mitotic figures in the Ehrlich ascites tumour following injection of Colcemid(R) at time zero. Untreated controls (★—★). Injection of 2 ml of Ehrlich ascitic fluid at zero hour (▲—▲). Injection of 2 ml of JB–1 ascitic fluid at zero hour (●—●). (See Fig. 5)

	No. of animals	Mean value of percentage of mitotic figures ± S.D.				
		0 hr	1 hr	2 hr	3 hr	4 hr
★–★	5	2·5 ± 0·3	3·7 ± 0·2	4·5 ± 0·2	5·7 ± 0·4	7·2 ± 0·4
▲–▲	6	2·4 ± 0·2	3·6 ± 0·1	4·7 ± 0·2	4·5 ± 0·3	4·5 ± 0·3
●–●	6	2·5 ± 0·3	3·5 ± 0·2	4·5 ± 0·2	5·8 ± 0·4	7·4 ± 0·7

S.D. = standard deviation.

Table 5. Percentage of mitotic figures in the JB–1 ascites tumour following injection of Colcemid(R) at time zero. Untreated controls (★—★). Injection of 2 ml of JB–1 ascitic fluid at zero hour (▲—▲). Injection of 2 ml of HB ascitic fluid at zero hour (●—●). (See Fig. 6)

	No. of animals	Mean value of percentage of mitotic figures ± S.D.								
		0 hr	½ hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr
★–★	4	2·3 ± 0·4	3·3 ± 0·4	3·9 ± 0·3	4·7 ± 0·5	6·0 ± 0·3	6·7 ± 0·3	7·4 ± 0·3	8·5 ± 0·2	9·4 ± 0·6
▲–▲	6		2·8 ± 0·3	3·9 ± 0·5	4·6 ± 0·6	5·8 ± 0·5	5·5 ± 0·6	5·5 ± 0·3	5·7 ± 0·5	6·9 ± 0·6
●–●	11		2·9 ± 0·3	3·9 ± 0·4	4·4 ± 0·3	5·7 ± 0·4	6·4 ± 0·9	7·6 ± 0·4	8·1 ± 0·6	9·2 ± 1·2

S.D. = standard deviation.

Table 6. Percentage of mitotic figures in the HB ascites tumour following injection of Colcemid(R) at time zero. Untreated controls (★—★). Injection of 2 ml of HB ascitic fluid at zero hour (▲—▲). Injection of 2 ml of JB–1 ascitic fluid at zero hour (●—●). (See Fig. 7).

	No. of animals	Mean value of percentage of mitotic figures ± S.D.								
		0 hr	½ hr	1 hr	1½ hr	2 hr	2½ hr	3 hr	3½ hr	4 hr
★–★	4	2·0 ± 0·1	3·0 ± 0·2	4·0 ± 0·3	4·4 ± 0·2	5·2 ± 0·3	5·2 ± 0·1	6·3 ± 0·3	7·0 ± 0·3	7·4 ± 0·1
▲–▲	3		2·4 ± 0·3	4·0 ± 0·5	4·5 ± 0·1	5·4 ± 0·2	5·8 ± 0·1	5·7 ± 0·2	5·7 ± 0·2	8·6 ± 0·4
●–●	6		2·5 ± 0·3	3·8 ± 0·5	4·4 ± 0·3	5·5 ± 0·1	6·0 ± 0·3	7·0 ± 0·4	8·5 ± 0·4	8·8 ± 0·6

S.D. = standard deviation.

Table 7. Labelling index ± S.D. of JB–1 and Ehrlich ascites tumours in recurrent growth determined 4 hr after injection of ascites fluid. In ( ) number of animals

	JB—1	Ehrlich
Non-injected controls	59±4 (6)	59±2 (5)
Addition of 2 ml of JB–1 ascites fluid	41±3 (7)	56±2 (4)
Addition of 2 ml of Ehrlich ascites fluid	61±4 (8)	36±6 (6)

S.D. = Standard deviation.

tumours in recurrent growth and the effect of cell-free JB–1 ascites on HB ascites tumours in recurrent growth (see legends and Tables 5 and 6).

It appears that cell-free ascites exert an inhibitory effect on the flux of cells into the mitotic phase if injected in mice bearing ascites tumours of the same origin. Furthermore, no inhibition of the G–2 phase is seen if the ascites tumours are treated with cell-free ascites from the "foreign" ascites tumour.

Previous experiments have demonstrated that injection of cell-free JB–1 ascites into mice bearing JB–1 ascites tumours in recurrent growth is followed by a decrease in the labelling index of the ascites tumour cells beginning 2 hr after the injection and lasting for about 5 hr. Almost the same effect has been demonstrated using Ehrlich ascites in Ehrlich ascites tumours in recurrent growth [6]. No decrease of the labelling index was seen 4 hr after injection of cell free Ehrlich ascites into AKA mice bearing JB–1 ascites tumours and vice versa (Table 7), supporting the idea that even the block in the presynthetic phase is a specific reaction.

DISCUSSION

Previous experiments have revealed that if acellular ascites obtained from mice bearing JB–1 ascites tumours in the plateau phase of growth is injected into mice bearing JB–1 ascites tumour in recurrent growth, the tumour cells are temporarily arrested in the presynthetic and in the G–2 phase.

In the present investigation simultaneous growth of JB–1 and Ehrlich ascites tumours in the same mouse was followed by a full and independent development of both tumours, the total number of tumour cells reaching a maximum of 2 to $2{\cdot}4 \times 10^9$, i.e. twice the number found after inoculation of one hypotetraploid ascites tumour.

The independent development of these two tumours indicates that some of the growth-limiting factors often claimed to be responsible for the decelerating growth rate of the ascites tumours, nutritional deficiency, metabolic waste, etc., are probably of minor importance. The JB–1 ascites tumour exerts no inhibitory influence on the Ehrlich ascites tumour cells in the mouse bearing both tumours and vice versa. Furthermore, injection of cell-free ascites into "foreign" ascites tumours in recurrent growth is not followed by a transient inhibition of the tumour cells in G–1 and G–2 which is otherwise seen when ascites of "own" origin is used.

The present data give further support to the hypothesis that the growth of ascites tumours is regulated by a humoral negative feedback mechanism similar to the normal growth regulation, and are, furthermore, consistent with the idea that the effector of this mechanism might prove to be a "chalone" [7–14], a substance known to regulate the proliferative activity of at least some normal tissues by means of tissue specific inhibition of the cells in the presynthetic and the mitotic phase.

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Viral Infections in Patients with Malignant Diseases

R. CAPPEL and J. KLASTERSKY

Service de Médecine et Laboratoire d'Investigation Clinique (Section des Maladies Infectieuses), Institut Jules Bordet, 1, rue Héger-Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1000 Bruxelles, Belgium

Abstract—*Infections caused by certain viruses appear to be more frequent in patients with malignant diseases.*

The present paper analyses the possible factors involved in the increased incidence of viral infections among patients with cancer and summarizes the therapeutic means of preventing or curing such infections.

CLINICAL manifestations of viral infections are usually short in duration and in most cases the offending viruses disappear rapidly from the site of the infection. The mechanisms by which the host eradicates these micro-organisms are still partly unknown. In some instances at least, mechanisms that are effective against bacteria might also operate against viruses. However, the effect of antibodies which are supposed to combine with the offending organism and to prevent its extension to the tissues is less active against viruses than against bacteria, since agammaglobulinemic children do not present more severe viral infections than normal children. Moreover, there is no clear relationship between the level of antiviral antibodies and the severity of many viral infections or the duration of the persistance of the viruses within the infected host. In chronic rubella, for example, the virus can still be isolated from the lens several years after the infection, despite high levels of circulating antibodies in the host [1]. A similar observation has been made in the aleutian disease of the mink which is caused by a virus [2]; here also the animal still harbors the virus despite high levels of circulating antibodies. This persistance might be explained by the formation of antibody–antigen complexes in which the virus would still remain infective; indeed if one treats the infective serum by antigammaglobulin antibodies, its infecting properties are significantly decreased [3]. Since viruses exert their pathogenic effects within the cells and because antibodies do not cross the cellular membrane it is not surprising that antibodies cannot, by themselves, eradicate viral infections, once they are established. However, it should be pointed out that the administration of antiviral antibodies is effective only before the phase of viremia, that is to say, before the target organ, where the virus will cause the lesion, is involved.

Once the viruses have infected the cells, mechanisms of defence other than antibodies must be considered to explain the resistance of the host. Among them interferon might play a role, as judged by experimental evidence. Interferon is a protein which is produced by cells which are infected by a virus, and in particular, by the leukocytes of the infected organism, very early during the infection. Its protective mechanism is not specific for a given virus, but interferon rather preserves the integrity of the cell against agression by many different viruses [4]. Interferon can be induced by substances other than the viral article itself; it has then some properties different from interferon induced by viruses [5], but its protective action against viral infection is maintained. The mechanisms by which interferon is protective are not completely elucidated; experimental data suggest that interferon produces within the cell the synthesis of a protein which inhibits the translation phenomenon (TIP) and blocks the formation of new

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viral RNA, whilst the synthesis of normal cellular proteins continues unchanged [6, 7]. The role of interferon in the protection against viral disease might be important to the clinician; it has been shown recently that herpes zoster was more severe in patients who were unable to produce large amounts of interferon [8]. However, it should be emphasized that in experimental animals, immuno-depression increases the severity of viral infections in spite of increased interferon production [9–11].

In patients with cancer, bacterial infection is a common cause of morbidity and mortality [12], but in these patients, especially those with lymphoma, viral infection can also be a frequent cause of disease.

It is important to notice that this kind of patient has a depressed antibody-producing function [13]. It would appear from this that the immuno-response might be of considerable importance in predisposing to severe viral infections those patients with malignant diseases. In patients with Hodgkin's disease about 13% of the infectious episodes are caused by viruses, but in lymphosarcoma and chronic lymphatic leukemia the frequence of viral infections seems to be lower. In these latter diseases the mechanisms of cellular immunity are probably less impaired than in Hodgkin's disease where severe impairment of cellular immunity and lymphopenia are common, especially during the terminal stages of the disease, that are most often associated with viral infection. Lymphopenia caused either by the neoplastic disease or the administration of chemotherapeutic agents such as corticoids might explain the susceptibility of the patient to viral infection by a reduced production of interferon or of cellular immunity [8]. Similarly, in patients who have undergone an organ transplantation and are being treated with immunosuppressive agents, the frequency of viral infections exceeds that seen in a normal population [14]. Therefore it seems probably that immunosuppressive therapy, which is common in the treatment of patients with cancer, might be an important factor predisposing such patients to infection by viruses. In addition to these iatrogenic factors, one must point out that cellular immunity is impaired in patients with cancer as compared with healthy people [15, 16] and that lymphocytes in patients with cancer have been found to be immunologically less effective than normal lymphocytes [17]. The role of cellular immunity in the resistance to viral infection has recently been reviewed [18].

Herpes zoster is the viral infection most frequently found in patients with cancer, especially those with Hodgkin's disease [19]; the frequency of this infection is comprised between 8 and 16% in patients with Hodgkin's disease, and that figure contrasts sharply with the 0·2–2% incidence found in healthy people. The disease usually appears in patients with cancer as a result of a reactivation of the virus, at the time when the immunological responsiveness of the host is severely impaired by the progression of the disease or the administration of immunosuppressive drugs [20]. Chickenpox, which is caused by the same virus as herpes zoster is also more frequent in patients with cancer, especially in children with acute leukemia, in whom the mortality of the disease may be important [21]. Patients with lymphoma are also more prone to present serious complications after vaccination against smallpox: generalized vaccinia and vaccinia necroticans are not uncommon complications in these patients, who should be vaccinated only for major reasons [22]. Therapy with methizazone may reduce the morbidity and the mortality of these complications and should be considered for therapy.

Clinically apparent infections caused by cytomegaloviruses are rare, although antibodies to this virus are extremely common in the general population. On the other hand immunosuppressive drugs, corticoids and hypogammaglobulinemia and diseases such as acute leukemia predispose frequently to cytomegalovirus infection. Seroconversion and recovery of the virus are more frequent under these circumstances [23] but clinical manifestations of the disease remain infrequent [24, 25]. Even in patients where disseminated cytomegalovirus infection can be demonstrated at autopsy, it is usually difficult to ascribe death to this infection. Similarly, in patients undergoing immunosuppressive therapy for organ transplantation, infection caused by cytomegalovirus is common [26]. However, cytomegaloviruria does not correlate with changes in renal function. Recovery of cytomegalovirus from the respiratory tract is often equivocal: most often other pathogens are associated with cytomegaloviruses and can explain by themselves clinical signs of pulmonary infection [26]. Therefore the role of cytomegaloviruses in infectious diseases of patients with cancer cannot be clearly established at the present time. It seems likely that most infections caused by the virus are subclinical and are not associated with serious morbidity or mortality.

The role of other viruses in the infectious complications of patients with cancer is almost

unknown. Measles may be particularly severe in children with leukemia [24, 28] and renal excretion of reoviruses in patients with Hodgkin's disease has been reported [29]. However, the significance of these observations and the mechanisms involved in their production remain to be established. It would be highly desirable that large prospective studies were undertaken to assess the role of viral infections in patients with cancer. The possible role of viruses in fever of unexplained etiology which is common in these patients remains to be investigated [30]. Similarly the role of associated viral infection in predisposing to bacterial disease [31], which is the main cause of mortality in patients with cancer, should be thoroughly evaluated, since therapeutic measures against viral disease are intensively investigated and might be of clinical use within the near future.

The protective effect of interferon against viral disease is well established in animals and has been found promising in limited clinical trials to prevent infection by influenza A2 virus [32]. In these trials interferon has been prepared from human leukocytes and administered intranasally. Obviously this mode of preparation would not provide sufficient amounts of interferon for regular clinical use. On the other hand, all the interferon-inducing agents available at the present time are highly toxic and cannot be used for therapeutic purposes. As already mentioned, the prophylactic role of human gammaglobulins against viral diseases, such as zoster, infectious hepatitis or measles, is established beyond doubt and can be used for prevention of these diseases after suspicious contacts with infected patients. During recent years special gammaglobulin preparations from hyperimmune human donors have become available for use. Hyperimmune gammaglobulin is now available for the treatment of vaccinial complications and for prevention of the complications of mumps. It may be suspected that these preparations will become more widely used in the management and prevention of various viral diseases. However, gammaglobulins cannot be of use to prevent viral diseases that result from activation of viruses already present within the host.

Most of the drugs which have been shown to possess inhibitory properties against viruses *in vitro* cannot be used in patients because of their toxicity [33]. Exceptions are amantadine, thiosemicarbazone, iododeoxyuridine and cytosine arabinoside. Amantadine prevents the penetration of influenza A2 virus into the cells and protects them against cytopathogenic lesions [34]. It has been shown effective in preventing epidemic influenza and encouraging results have been reported in the treatment of subacute sclerosing encephalitis [35]. As already mentioned methizazone is effective in the treatment of infections caused by vaccinia [36] and to prevent smallpox if administered soon enough after the infection.

Iodouridine and cytosine arabinoside inhibit the synthesis of DNA. Successful therapy of patients with herpetic encephalitis with iodouridine has been reported [37] as well as therapeutic efficacy in cutaneous and ocular herpetic infections. Cytosine arabinoside, which is active *in vitro* against herpes viruses, might be effective in severe zoster and varicella infections, as well as in pneumonia caused by cytomegaloviruses [38, 39]. More recently derivatives of rifampicin have been described as highly effective against several viruses *in vitro* at concentrations that could be attained in the human being without excessive toxic effects; intensive investigation of these compounds is at present performed in many laboratories and will lead presumably to some clinical application [40, 41].

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Experiments Aiming at Demonstrating Microsomal Drug Metabolism in the Tumor Tissue

MARIA GRAZIA DONELLI, SANTINA COLOMBO and SILVIO GARATTINI
Istituto di Recerche Farmacologiche "Mario Negri", 62, Via Eritrea, 20157 Milano, Italy

Abstract—*Besides the liver, other organs such as lung, kidney, and placenta possess microsomal enzymes. Since several antitumoral drugs are metabolized by this enzyme system, it was of interest to investigate whether the microsomal fraction of the tumor tissue was able to metabolize foreign compounds.*

*The present paper shows that within the sensitivity of the method, 9000 × **g** and 105,000 × **g** fractions of various experimental tumors of the rat and the mouse do not metabolize known substrates of the microsomal enzymes such as p-nitro-anisol and aniline. Lack of metabolism was also observed by increasing the amount of tumor tissue employed, the amount of cofactors and the incubation time.*

Neither pretreatment of the tumor-bearing animals with phenobarbital or 3-methyl-cholanthrene induces metabolization.

IT IS WELL known that a variety of drugs are metabolized by enzymatic systems localized in liver microsomes in the presence of NADPH and oxygen [1, 2]. Besides the liver, other organs such as lung, kidney and placenta possess microsomal enzymes, although to a low extent, as compared to the liver [3]. Since no microsomal metabolism has ever been described for the tumor tissue and since several antitumoral drugs are metabolized by this enzyme system [4–6] it was of interest to investigate whether the microsomal fraction of the tumor tissue was able to metabolize foreign compounds.

This could have a great bearing with regard to the antitumoral agents which are stored in this tissue. This paper reports studies which were undertaken to elucidate this problem.

Male Sprague–Dawley rats (150 ± 5 **g** body weight), female Albino Swiss mice (20 ± 1 **g** body weight), male C57 Bl/10 J inbred mice (22 ± 1 **g** body weight) and male or female ($C_3H \times 020)F_1$ inbred mice were used. Animals had free access to food (diet ALAL 56) and water during the experiments.

All animals were kept at room temperature of 22°C with a relative humidity of 60% in cages of Makrolon (20 × 36 × 13 cm for 10 mice, and 47 × 25 × 15 cm for 6 rats).

Walker 256 carcinosarcoma, T 8 Guérin epithelioma and Sarcoma 45 were transplanted subcutaneously (about 100 mg of a 15-day old tumor) by means of a trocar in the right flank of Sprague–Dawley male rats.

Ehrlich carcinoma and Sarcoma 180, maintained in ascites form for more than 300 serial passages were transplanted intramuscularly or intraperitoneally in female Albino Swiss mice.

Only ascitic fluids obtained from mice inoculated 7 to 10 days before were used, in order to keep cell clumps and dead cells to a minimum. For intramuscular and intraperitoneal injections 5,000,000 tumor cells were suspended in 0·2 ml buffer phosphate.

Lewis Lung carcinoma, maintained in ascites form for more than 50 serial passages, was transplanted intramuscularly in male C57

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Bl/10 J mice. A quantity of 0·1 ml ascitic fluid taken from mice inoculated 20 days before and diluted 1/50 with phosphate buffer (pH 6·9) was injected into each mouse.

($C_3H \times 020$) F_1 hybrid mice bearing a mammary adenocarcinoma induced in female by force-breeding and in castrated males by treatment with oestrogens were obtained from the International Center for the Provision and the Study of Tumor-bearing Animals (Mühlbock, Amsterdam, The Netherlands).

The experiments were performed 16 or 17 days after the subcutaneous transplantation of Walker carcinosarcoma, T 8 uterine epithelioma of Guérin and Sarcoma 45 in the rat, 16 days after the intramuscular and 12 days after the intraperitoneal transplantation of Sarcoma 180 or Ehrlich carcinoma in the Swiss mouse, 15 days after the intramuscular transplantation of Lewis Lung carcinoma in the C57 Bl/10 J mouse and for the mammary adenocarcinoma when the ($C_3H \times 020$) F_1 mice were about 37 + 2 **g** body weight.

The metabolizing capacity of the above mentioned tumor tissues was investigated *in vitro* by incubating at 37°C tumor tissue fractions with *p*-nitro anisol and aniline which are known substrates of the microsomal enzymes and by determining their metabolites, *p*-nitrophenol and *p*-aminophenol respectively [7].

The experimental conditions used were those employed for testing the *in vitro* microsomal metabolism of the liver. The tumor tissue was removed immediately after sacrifice, the necrotic part discarded when possible and after homogenization in 4 volumes of ice-cold 1·15% KCl solution in a Potter-type Teflon homogenizer, centrifugation was performed at 9000 × **g** for 20 min. The supernatant fraction was ultracentrifuged at 105·000 × **g** in order to obtain the microsomal pellet. Three ml of 9000 × **g** supernatant (equivalent to 600 mg tissue) or microsomes (equivalent to 1 **g** tissue) suspended in 2 ml ice-cold 1·15% KCl solution were mixed with 2 ml of a solution containing NADP (1·5 μmoles), glucose-6-phosphate (50 μmoles), magnesium chloride (25 μmoles), nicotinamide (50 μmoles), 280 μmoles potassium phosphate buffer (pH 7·4) and the substrate (3 μmoles *p*-nitro anisol and 5 μmoles aniline).

When microsomes were employed, 0·5 units of glucose-6-phosphate dehydrogenase were added to each incubation flask.

Table 1. *Protein content of mouse and rat tumors and liver*

Tumor or tissue	Site of implant	Animal species	Proteins (mg/g ± S.E.) Homogenate	9000 × **g**	105,000 × **g**
Walker carcinosarcoma	Subcutaneous	Sprague–Dawley rat	92·5 ± 1·6	56·8 ± 1·1	3·8 ± 0·1
T 8 epithelioma of Guérin	Subcutaneous	Sprague–Dawley rat	91·2 ± 2·7	63·2 ± 1·9	10·5 ± 0·9
Sarcoma 45	Subcutaneous	Sprague–Dawley rat	96·2 ± 4·4	68·3 ± 2·6	12·1 ± 0·5
Sarcoma 180	Intramuscular	Swiss-Albino mouse	81·2 ± 3·8	58·1 ± 2·5	8·4 ± 0·4
Sarcoma 180	Intraperitoneal	Swiss-Albino mouse	68·6 ± 1·8	35·8 ± 1·3	0·35 ± 0·01
Ehrlich carcinoma	Intramuscular	Swiss-Albino mouse	96·9 ± 2·3	63·0 ± 1·3	7·9 ± 0·3
Ehrlich carcinoma	Intraperitoneal	Swiss-Albino mouse	75·1 ± 2·2	37·6 ± 1·1	0·29 ± 0·01
Lewis Lung carcinoma	Intramuscular	C57 Bl/10 J mouse	128·8 ± 6·3	77·7 ± 3·6	7·4 ± 0·9
Mammary adenocarcinoma	Spontaneous	($C_3H \times 020$) F_1 mouse	116·7 ± 3·4	72·9 ± 4·4	4·6 ± 0·4
Liver		Sprague–Dawley rat	215·5 ± 3·8	151·6 ± 0·4	16·2 ± 0·3
Liver		Swiss-Albino mouse	219·6 ± 8·3	129·3 ± 5·1	13·0 ± 0·8

The mixture was then incubated at 37°C for 30 min in the open air in a Dubnoff metabolic shaker. Since these experimental conditions could be not the best ones, some modifications in the procedure were introduced in order to increase the microsomal metabolism of the tumor tissue (9000 × **g** and 105·000 × **g** fractions of subcutaneous Walker carcinosarcoma for the rat and of intramuscular Ehrlich carcinoma for the mouse):

(1) The amount of tumor tissue employed was increased up to 1 **g** for the preparation of 9000 × **g** and 2 **g** for the preparation of 105·000 × **g** fraction.

(2) Tumor homogenates were incubated with a double amount of cofactors.

(3) Thirty min after the beginning of the incubation additional 1·5 μmoles of NADP were added.

The determination of the metabolites, *p*-nitrophenol and *p*-aminophenol produced respectively by *p*-nitro anisol and aniline was performed according to the method of Gilbert and Goldberg [7]. The protein content of the tumor tissue was determined according to Lowry [8].

Within the sensitivity of the method which is about 15 mμmoles for both compounds these tumor tissues do not show any metabolization (less than 0·6%) of the substrates being tested under any of the described experimental conditions, whereas the rat liver metabolizes about 173 ± 14 mμmole *p*-nitro anisol and 245 ± 26 mμmole aniline/**g** liver ($9000 \times$ **g** fraction), 107 ± 10 mμmole *p*-nitro anisol and 154 ± 11 mμmole aniline/**g** liver ($105{,}000 \times$ **g** fraction), and the mouse liver $742{\cdot}7 \pm 16$ mμmole *p*-nitro anisol and $1{,}110{\cdot}6 \pm 44$ mμmole aniline/**g** liver ($9000 \times$ **g** fraction), $418{\cdot}5 \pm 18$ mμmole *p*-nitro anisol and 562 ± 25 mμmole aniline/**g** liver ($105{,}000 \times$ **g** fraction).

The protein content of the various tumor tissues and liver fractions is indicated in Table 1.

Since compounds like phenobarbital and methylcolanthrene considerably increase the liver microsomal enzymes activity, experiments were run where tumor tissues microsomes taken from rats and mice pretreated for 2 days with phenobarbital (30 mg/kg/i.p.) or with methylcolanthrene (20 mg/kg/i.p.) were tested in their metabolizing capacity. Also in these experimental conditions no drug metabolism was observed. Furthermore in order to answer the question whether the tumor tissue possesses some inhibitors of the microsomal metabolism, an experiment was carried out where 1 ml of tumor homogenate (1/5) was added to flasks containing incubation mixtures with $9000 \times$ **g** liver fractions.

The amount of substrates metabolized in this case was not significantly different from that obtained without addition of the tumor homogenate.

The findings above reported indicate that at least under the experimental conditions described the tumour tissue is not provided with the enzymatic systems for drug metabolism present in the endoplasmic reticulum of the liver.

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Handbook of Materials and Methods

E.O.R.T.C. SCREENING GROUP*†
Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy

E.O.R.T.C. SCREENING PROCEDURES

Group A—substances of unknown action or which are available only in small amounts

A_1 KB cells (4)‡
A_2 Physarum polycephalum (8)
A_3 DNA or RNA viruses (7)

Group B—compounds already shown to be effective in vitro *or analogues of known anti-tumour drugs*

B_1 Leukaemia L 1210 (6)
B_2 Ehrlich adenocarcinoma (4)

Group C—chemicals made for special purposes or compounds effective in groups A or B

C_1 Stem cell kinetics (9) (11)
C_2 Haemolytic plaque assay (1)
C_3 Myeloma MOPC (6)
C_4 Melanoma 91 (6)
C_5 Walker 256 (6)
C_6 Sarcoma BP 8 (6)
C_7 Graft versus host (10)
C_8 Sarcoma 180 (4)
C_9 Spontaneous mammary tumour (4)
C_{10} Cancer cell dissemination and metastasis (4)
C_{11} Haematological tests (3)
C_{12} Friend-leukaemia virus (7)
C_{13} Spectrum of activity in various tumours (2)
C_{14} Carcinogen-induced, hormone dependent carcinoma of the rat (5)

A_1 KB CELLS *IN VITRO* [1]

After trypsinization, 50,000 cells in 1 ml of growth medium are seeded in each culture tube. The tubes are incubated at 36°C in a roller apparatus at 6 rev/min for 48 hr. Thereafter, a few tubes are trypsinized and counted by an electronic cell counter (base line = N_{LB}), and others are treated by changing the growth medium. After a further 48 hr incubation the remaining cells are trypsinized and counted. From the value obtained (N_{48}), the growth rate of cultures, both control (N_{cc}) and treated (N_{et}) may be determined by the following equation:

$$\frac{N_{48}}{N_{LB}} - 1 = N_{cc}\,.$$

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*Compiled by G. Franchi, with the secretarial assistance of Miss S. Gönczy.

†Members:

1. Amiel, J. L. (Institut de Cancérologie et d'Immunogénétique, Paris, France).
2. Connors, T. (Chester Beatty Research Institute, London, Great Britain).
3. Fliedner, T. M. (Zentrum für Klinische Forschung, Universität Ulm, Ulm, Germany).
4. Garattini, S. (Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy).
5. Heuson, J. C. (Institut Jules Bordet, Bruxelles, Belgium).
6. Mathé, G. (Institut de Cancérologie et d'Immunogénétique, Paris, France).
7. Munk, K. (Institut für Virusforschung, Heidelberg, Germany).
8. Sachsenmaier, W. (Institut für Experimentelle Krebsforschung, Innsbruck, Austria).
9. Stryckmans, P. (Institut Jules Bordet, Bruxelles, Belgium).
10. van Bekkum, D. W. (Radiobiological Institute TNO, Rijswijk, Netherlands).
11. van Putten, L. M. (Radiobiological Institute TNO, Rijswijk, Netherlands).

‡In brackets are the participating members responsible for the test.

Growth inhibition is determined as a percentage of control growth:

$$\% \text{ inhibition} = 100 - \frac{N_{et}}{N_{cc}} 100$$

The results are classified as follows:
very active compounds (+ +),
100% inhibition at a dose of 1γ/ml;
active compounds (+),
100% inhibition at a dose of 10γ/ml;
inactive compounds (0),
inhibition at a dose of 100γ/ml or more.

A_2 PHYSARUM POLYCEPHALUM *IN VITRO*

(1) *Growth inhibition of Physarum polycephalum (stationary macroplasmodia)*

Principle. Growth inhibitory action of substances is evaluated semi-quantitatively by comparing the increase of the surface area of treated and untreated disk-shaped macroplasmodia over a period of 48 hr.

Procedure. A series of macroplasmodia of Physarum polycephalum is prepared from exponentially growing suspension cultures by fusion of a large number of microplasmodia on filter paper.

Test substances are added to the liquid medium at a final concentration of 200 μg/ml. Water-insoluble compounds (6 mg) are dissolved in 0·1 ml ethanol, acetone or dimethylsulphoxide plus 0·1 ml Tween 40 and subsequently diluted with nutrient medium. Two cultures with normal medium serve as controls. All cultures are incubated for 48 hr at 26°C.

Evaluation. The diameters of the disk-shaped macroplasmodia are measured after 24 and 48 hr of incubation. Substances which inhibit the increase of the plasmodial size (surface area) by less than 50% are considered inactive. All other compounds are tested further at various concentrations in suspension cultures (test 2).

(2) *Inhibition of protein synthesis of Physarum polycephalum (supension of microplasmodia)*

Principle. Growth inhibitory action of substances is evaluated by the quantitative measurement of protein synthesis in suspension cultures of Physarum polycephalum using a modified procedure of J. Becker *et al.* [2].

Procedure. Microplasmodia are isolated by centrifugation (2 min at 500 × **g**) of 40 ml of an exponentially growing suspension culture and are re-suspended with 3 times the sediment volume of fresh liquid medium. Next 1·5 ml aliquots (~ 8 mg protein) are pipetted into a series of 100 ml Erlenmeyer flasks containing 7·5 ml medium. Two aliquots of the inoculum are centrifuged immediately and stored in the freezer. Test substances are added to the flasks as a solution or suspension in 1 ml medium to give final concentrations of 0, 10, 100 and 200 μg/ml (two flasks per dose).

All cultures are agitated on a reciprocal shaker at 120 rev/min at 23°C for 24 hr and subsequently centrifuged (2 min at 500 × **g**). The sedimented microplasmodia are re-suspended with distilled water (final volume 4 ml), mixed with 4 ml ice-cold 0·5 N trichloroacetic acid (TCA), stored in ice for 15 min and centrifuged. The pellets are washed (by re-suspending and centrifugation) 2 times each with 4 ml 0·25 N TCA, 8% TCA in acetone, and again with 0·25 N TCA. Finally the pellets are dissolved with 20 vol. 0·4 NaOH at room temperature overnight and the proteins are determined by the Folin method [3].

Evaluation. The increase of the protein content over the inoculum value is calculated as a percentage of the corresponding increase in the controls (mean deviation of duplicates: ±15%). The inhibitory action of a drug is characterized by the (substance) concentration which inhibits protein synthesis by 50% (ED_{50}). The results are classified as follows:

ED_{50}	> 200 μg/ml	(0)
	10–200 μg/ml	(+)
	< 10 μg/ml	(+ +)

A_3 DNA VIRUS: HERPES VIRUS HOMINIS
RNA VIRUS: MEASLES VIRUS

Virus infections lead to either cell death or neoplastic transformation of the cell. The cytolytic effect is the direct result of viral multiplication, while the oncogenic activity is due to the direct or indirect functions of an integrated viral genome within the transformed cell. The study of the antiviral activity of chemical substances should be related to the inhibition of these specific viral activities [4, 5].

The cytolytic activity can be tested *in vitro* using the cytopathic effect caused by the virus in cell cultures. In addition, the reduction of the amount of virus produced by the cell in tissue cultures can be used as a more specific parameter.

Procedure

The *in vitro* screening is performed by using the DNA-containing Herpes virus hominis (HVH) and the RNA-containing Measles virus. The HeLa cells serve as host cells. Both viruses have been chosen for this test because of their relatively long multiplication cycle.

The "agar-diffusion, plaque-inhibition test". This has been described by Herrmann *et al.* [6] and Rada *et al.* [7], and has been chosen for the general screening test method in this programme. HeLa cells are seeded into plastic petri dishes. These cultures are infected with HVH or Measles virus in concentrations that produce an appropriate number of plaques. One hour after infection the nutritional agar overlay is added to the cell culture. A small round piece of filter paper is placed on this agar overlay, and the compound to be tested is added, on the filter paper, in an appropriate amount (200 μg). Six days after infection the cell cultures are vital-stained in order to show the plaques which have formed and to quantitate the results on the 7th day. One can detect toxicity of the drug by examination of the zone of unstained cells around the filter paper containing the drug. *Antiviral activity is indicated by a large zone of neutral red-stained plaque-free cells.* This screening test allows one to compare the toxic zone with the plaque-free zone and to demonstrate the activity of the substance [8].

The results obtained are classified as follows:

DNA Virus plaque inhibition (+)
no inhibition (−)

RNA Virus plaque inhibition (+)
no inhibition (−)

B_1 L 1210 LEUKAEMIA

The leukaemia tumour used for this test is L 1210. It is a lymphoblastic leukaemia chemically induced by 3-methyl-cholantrene, in DBA/2 mice. This leukaemia is transplanted by graft into DBA/2 or into hybrids of DBA/2. It exists in two forms:

an ascitic form in which leukaemia cells are injected intraperitoneally, or

a solid form in which the injection is performed subcutaneously.

The solid form of the leukaemia is used for the trials. In the experiments DBA/2 hybrids are used. The trial is performed in 3 stages.

1st test: Evaluation of the toxicity of the compound

Since the acute LD_{50} is unknown, it is determined approximately. The drug is administered to mice at doses of 5, 25, 125 and 625 mg/kg (2 animals for each dose). Two normal mice of the same strain are used as controls. After observing the number of deaths occurring in less than 10 days at different doses, Table 1 is employed to establish the doses for the second test.

Table 1

	Preliminary Toxicity (mg/kg)				Doses to be used with L 1210 (mg/kg)		
	625	125	25	5			
1	0	0	0	0	1500	600	240
2	1	0	0	0	1250	500	200
3	2	0	0	0	625	250	100
4	2	1	0	0	250	100	40
5	2	2	0	0	125	50	20
6	2	2	1	0	50	20	8
7	2	2	2	0	25	10	4
8	2	2	2	1	10	4	1·6
9	2	2	2	2	5	2	0·8

2nd test: Simultaneous evaluation of the reliability of the acute LD_{50} test and measurement of the efficacy of the compounds on L 1210

Each mouse receives 5×10^4 leukaemic cells subcutaneously in the axillary region. Animals are divided at random into as many groups as the drugs to be tested, and one group is left as a control.

The day after the injection of tumour cells the drugs are injected into the animals subcutaneously if they are water-soluble, or intraperitoneally if liposoluble. The 3 doses used are in a geometric series with a ratio of 2 : 5 (see Table 1) and are obtained from the results of the first test. For each dose five animals (15 for each drug) are used and the control group receiving only the L 1210 leukaemia is composed of 5 $\sqrt{k}$ animals, k being the number of drugs to be tested in the same trial. Another group of animals is treated with known active antitumour drugs a day after tumour cell injection (Vinblastine, 6MP or Rubidomycine).

Thus, animal survival time can be compared either to the control group or to an established active drug.

3rd test: Confirmation of results

(*a*) *Estimation of acute LD_{50}.* Evaluation is performed on the basis of Karber's formula. It is also possible to employ a graphic method

where the percentage of deaths, due to the toxicity at each dose, is shown on the ordinate in an arithmetic scale, while the administered doses are indicated on the abscissae in a semi-logarithmic scale. The point on the line corresponding to 50% mortality gives the LD_{50}.

(*b*) *The effect of L* 1210. The survival time of treated animals is compared to that of control animals following the unparametric test of Wilcoxon's W, since mouse deaths are not distributed according to a normal distribution. Accordingly, a rank is given for each animal's death—the first death receives rank 1, the second rank 2, etc. The sum of the observed ranks of the control animals $\sum_c$ is calculated, as well as the ranks of the treated animals $\sum_t$.

$$\sum_c = \frac{n_1(N+1)}{2} .$$

The variance of the addition of the orders is:

$$s^2 = \frac{n_1 n_2 (N+1)}{12} ,$$

where n_1 is the number of control animals, n_2 is the number of treated animals, N is the total number of animals.

According to the ratio:

$$\sum = \frac{\sum_t - \sum_c}{\sqrt{s^2}}$$

the value given in the table (Wilcoxon's W) indicates the statistical significance.

B_2 EHRLICH ADENOCARCINOMA

(1) *Preliminary toxicity in normal animals*

Doses to be used (mg/kg i.p.): 5, 25, 125 and 625. For each dose 2 animals are used. The treatment consists of a single injection. The evaluation is made 10 days afterwards. The LD_{50} is calculated by the Spearman–Karber method [9]. From the results of the preliminary toxicity study 3 doses are chosen for therapeutic tests:

(2) *Evaluation of anti-tumour effect*

Ehrlich carcinoma is transplanted (5,000,000 cells/0·2 ml) subcutaneously in Swiss female mice. For each dose 5 animals are used. The animals are treated 1 day after tumour transplantation. The evaluation of the drug effect is performed 12 days after tumour transplantation. The mean weight of the whole body, of the tumour and spleen and the mean number of circulating leucocytes of treated animals are compared with the controls using the *t* Student's test.

Preliminary Toxicity (mg/kg i.p.)				Doses chosen for therapeutic test		
625	125	25	5			
0	0	0	0	1600	800	400
+	0	0	0	320	160	80
+	+	0	0	80	40	20
+	+	+	0	16	8	4
+	+	+	+	3·2	1·6	0·8

+ Indicates at least one death.

C_1 STEM CELL KINETICS [10–12]

For selected drugs, more information may be sought on the mechanism of action. Since many drugs act preferentially on rapidly growing cells or on cells in a particular phase of the cell cycle, for example only during mitosis, as with the spindle poisons or only during DNA synthesis, tests can be performed to obtain information on this point.

1. For this purpose hemopoietic colony-forming stem cells are exposed to the agents under different conditions, namely: (*a*) normal "resting" cells in mouse bone marrow, and (*b*) rapidly proliferating stem cells in the spleens of irradiated mice that had been injected with a standard number of bone marrow cells. In (*a*) the number of colony-forming units in the femur bone marrow is determined 16 hr after injection of various doses of the drug; in (*b*) the number of colony-forming units in the spleen of irradiated bone marrow treated animals is determined 7 days after irradiation, and 16 hr after injection of various doses of the drug.

From the dose–effect curves under these conditions, the existence of differences in sensitivity between resting and rapidly proliferating cells may be evaluated. This gives information on the degree to which the stem cell sensitivity to the drug is dependent on stem cell kinetics. In some cases, additional studies are performed in which the survival of leukaemic cells (L 1210 leukaemia) or the cells of a solid tumour (mouse osteosarcoma C 22 LR) after different doses of the drug is evaluated. For a number of drugs it appears that the tumour cell killing is greater than can be explained on the basis of tumour cell kinetics, indicating that different mechanisms are involved.

2. In human acute leukaemia, the bone marrow leukaemic cells are generally more actively proliferating than the blood leukaemic

cells. This provides an experimental situation in which the population of leukaemic cells of one patient is composed of two fractions located in different parts of the body and proliferating at different rates.

The technique consists of withdrawing on day 1, 0·5 l. of blood from a leukaemic patient. The leukaemic blasts are labelled *in vitro* with ^{3}H Cytidine for 1 hr. The blood is then autotransfused within 10 min and the labelled blasts are followed for 7 to 10 days on autoradiographs of serial blood samples. The drug to be tested is administered on day 3 or 4. The absolute number per mm^3 and the percentage of labelled blasts is determined prior to and after the administration of the drug. These values decrease exponentially with time.

The slope of the absolute number of labelled blasts per mm^3 is a function of the exit of leukaemic cells from the blood, namely the number of cells leaving the blood to the tissues per unit of time plus the number of cells dying per unit of time. This method permits one to detect a cytocidal effect of a drug on the slowly proliferating leukaemic blasts in the blood which are mainly in G_1 phase of the cell cycle.

The slope of the per cent of labelled blasts is a function of the number of blasts entering the blood per unit of time, that is to say, the input from the bone marrow. The effect on the slope of the drug which is tested, permits one to determine whether it is more, less, or equally active on bone marrow than on blood leukaemic blasts.

C_2 HEMOLYTIC PLAQUE ASSAY (JERNE'S TEST)

This test is performed to screen drugs considered as modifiers of the immune response.

Materials and methods

Sheep erythrocytes are used as antigens. The erythrocytes are washed three times, by centrifugation. The residual cells are diluted (1 : 10) in saline.

DBA/2 or C57/Bl/6 mice, 3–5 months old, are used. Eighteen animals are used for each drug. They are divided at random into 3 groups of six animals each. Two groups are treated with a single injection, the third one is kept for the control.

On day 0, 0·5 ml of the 1 : 10 diluted sheep erythrocytes is injected into each animal. Four days before antigen administration, the first experimental group receives the drug at a dose of 1/3 of the acute LD_{50}, intraperitoneally or subcutaneously according to its water or lipid solubility. The day after the antigen injection, the second experimental group receives the same drug at the same dose and by the same route. The third control group receives only the antigen 4 days after injection animals are sacrificed. A cell suspension from each spleen is prepared, counted and diluted to 10^7 living spleen cells /ml. A volume of 0·1 ml of sheep red cells diluted to 1/5 is put into a tube containing an agar solution.

The contents of each tube are poured into a petri dish. Three petri dishes are used for each spleen. The dishes are incubated at 37°C. An hour later, diluted guinea pig serum is added to all the dishes which are then put into the incubator for an additional 30 min. At the end of this period, around each antibody-producing cell a clear, haemolytic plaque is formed. The haemolytic plaques in each dish are counted.

Evaluation of the results

The mean number of haemolytic plaques for each spleen and for each group treated are compared to the controls using the *t* Student's test. The drug is to be considered adjuvant when the average number of haemolytic plaques in the treated group is significantly higher than in the controls. On the contrary, the drug is an immunodepressant when the average number of haemolytic plaques is significantly lower than in the controls.

C_3 MYELOMA MOPC

This test is performed only if the drug proves to be active on leukaemia L 1210, or if its chemical composition (eg. derivatives of phenylalanine) suggests that it might act on the myeloma.

Materials and methods

The MOPC myeloma is maintained in mice of the DBA/2 strain by a subcutaneous transplantation of a tumour fragment into the axillary region. For the therapeutic test 2 and 4 month-old DBA/2 mice receive 5×10^6 tumour cells subcutaneously in the axillary region. Ten animals are used for each drug. The treatment consists of a single injection made the day after the tumour implantation.

Evaluation of the results

The survival times of treated and control animals are compared. Wilcoxon's W unparametric test is used for statistical evaluation. Since the death of mice is not distributed

according to a normal distribution, a rank is given to each mouse death, and the treated animals' ranks are compared to those of the controls. The drug is considered active when the difference in survival time between the two groups of animals is significant ($\sum$ is higher than that of Wilcoxon's table).

C_4 MELANOMA S 91

This test is not systematically performed for all the drugs submitted to chemotherapeutic screening. It is performed only when drugs prove to be effective on leukaemia L 1210.

Materials and methods

The S 91 melanoma is transplanted into DBA/2 or into hybrids of DBA/2 mice. On the day of the experiment all the animals are injected intraperitoneally with 10^7 tumour cells. The mice are divided at random into K groups of n animals (K being the number of substances to be tested). The control group consists of $n\sqrt{K}$ animals.

The next day, all the animals of the treated groups receive a single injection of the drugs subcutaneously or intraperitoneally at a dose corresponding to 1/3 of their LD_{50}. Eighteen days later, all the animals are sacrificed and the tumours excised and weighed.

Evaluation of the results

The average tumour weight for each treated group is compared to that of the control animals using the Student–Fischer t test. The drug is active when it significantly decreases the tumour weight in treated animals.

C_5 WALKER 256 CARCINOSARCOMA

This test is performed only if the drug proves to be effective on leukaemia L 1210 or on Ehrlich carcinoma or if its chemical composition suggests that it might act on the carcinoma (eg. nitrogen mustards, alkylating agents).

Materials and methods

Walker 256 carcinosarcoma is transplanted into 6 or 8 week-old Albino rats. This tumour grows rapidly and soon kills the host animal.

Male CF rats in a number equal to $6(K+\sqrt{K})$, where K is the number of compounds to be tested, receive subcutaneously 2×10^6 tumour cells in the axillary region. They are divided at random into groups of 6 animals each, and a group of $6\sqrt{K}$ animals is left for controls.

One day after the tumour injection 6 rats of each group under treatment are treated with a single injection of the drug at a dose of 1/3 of the acute LD_{50}. The drug is administered subcutaneously if it is water-soluble and intraperitoneally if it is lipid-soluble. On the ninth day after tumour injection all the animals are sacrificed and the tumours are excised and weighed.

Evaluation of the results

The average tumour weight for each treated group is compared to that of the controls according to the Student–Fischer t test. If the drug significantly decreases the tumour weight of treated animals in comparison to that of the controls it is considered active.

C_6 SARCOMA BP 8

This test is performed only if the drug shows some activity on leukaemia L 1210 or on Ehrlich carcinoma.

Materials and methods

The BP 8 is an ascitic tumour, chemically induced in C3H mice. It is intraperitoneally transplanted from one animal to the other by injecting a small volume of diluted ascitic fluid. In each experiment 10 animals per compound are used and $10\sqrt{K}$(K being the number of compounds tested submitted to the experiment) as control animals.

On the day of the experiment all the mice (C3H/Eb, 2 and 4 months old) receive 10^6 BP 8 ascitic cells intraperitoneally. Later on, animals are divided at random into a control group and into treated groups which receive a single injection of the different drugs the day after the tumour injection at a dose of 1/3 of the LD_{50}. The drugs are administered subcutaneously or intraperitoneally depending on their solubility or insolubility in water.

Evaluation of the results

Animal survival time is observed. Treated animals are compared with the controls, according to the unparametric Wilcoxon's W test. When $\sum$ calculated in the experiment is higher than that of the table, the survival time difference is significant and the drug is active.

C_7 GRAFT VERSUS HOST REACTION

Purpose

Homologous bone marrow transplantation in irradiated primates is followed by an acute graft vs. host reaction (called acute or early secondary disease) which runs a uniformly lethal course when unrelated bone marrow donors are employed. This reaction is due to a rapid proliferation of the lymphatic components of the graft and a violent immunological reaction of these cells against the tissues of the host.

One approach is to inhibit or prevent this reaction by the administration of cytostatic agents, the problem being that the suppression of the transplanted immunologically active cells has to exceed the suppression of the haematopoietic cells, otherwise the treatment will result in death from bone marrow aplasia. This differential effect may be a property of certain drugs (immuno-suppressive agents) or be dependent on the *dose* and/or time of administration (dosage schedule).

In the monkey the number of different dosage schedules that can be investigated is obviously extremely limited. Therefore a screening system was developed in mice, which permits the testing of a very large number of different dosage schedules in a short time and at comparatively low cost.

Description of the test

The test was developed from a screening system for the investigation of selective elimination of immunologically active cells *in vitro* in a mixture of lymphatic and haemopoietic cells [13]. This test in mice consists of the i.v. administration of 2×10^6 eosin-resistant CBA spleen cells to F_1 (CBA × C57BL) hybrids which have been exposed to whole body irradiation with a dose of 912 R (875 rads). This amount of spleen cells causes *acute* secondary disease which kills the recipients within 8 days. In this respect it simulates the situation in the primate where the most severe cases of acute secondary disease following homologous bone marrow transplantation die around that day.

The 2×10^6 spleen cells, however, contain a sufficiently large number of haemopoietic cells to guarantee recovery of the haemopoiesis of the irradiated mice, and consequently their survival if the graft versus host reaction were prevented. In this test, therefore, survival time exceeding 20 days is proof of selective suppression of the proliferation of the immunologically active cells. If the mice die within that period it is easy to distinguish between death from the acute secondary disease (meaning that the drug was ineffective) or from haemopoietic failure (meaning that the drug was not selective) by microscopic examination of the bone marrow, and the clinical symptoms may provide similar information.

C_8 SARCOMA 180

(1) *Preliminary toxicity in normal animals*

Doses to be used (mg/kg i.p.): 5, 25, 125 and 625. For each dose 2 animals are used. The treatment is continued for 6 days. The evaluation is made 12 days after the beginning of the treatment. The LD_{50} is calculated by the Spearman–Karber method [9].

From the results of the preliminary toxicity 3 doses are chosen for therapeutic tests.

Preliminary toxicity (mg/kg i.p.)				Doses chosen for the therapeutic test		
625	125	25	5			
0	0	0	0	1600	800	400
+	0	0	0	320	160	80
+	+	0	0	80	40	20
+	+	+	0	16	8	4
+	+	+	+	3·2	1·6	0·8

+ Indicates at least one death.

(2) *Evaluation of antitumour effect*

Sarcoma 180 is transplanted (5,000,000 cells/0·2 ml) subcutaneously into Swiss female mice. For each dose 10 animals are used. The treatment begins 5 days after tumour transplantation and is continued for 6 days. The evaluation of the drug effect is performed 2 days after the last treatment. The mean weight of whole body, tumours and spleens, and the mean number of circulating leucocytes of treated animals are compared to the controls using the *t* Student's test.

C_9 SPONTANEOUS MAMMARY TUMOUR

$(C_3H \times O_{20})F_1$ mice, male castrated and treated with oestrone in drinking water (2 mg/l.) and female "force breed" develop carcinoma of the mammary gland. In both groups the tumour incidence is nearly 100% at an average age of 254 and 248 days, respectively. The mice are supplied by Prof. O.

Mühlbock from the National Cancer Institute, Amsterdam. At arrival two diameters of the tumours are measured and tumour growth observed for about a week.

After this lapse of time those mice which do not show any change in tumour volume are excluded from the experiment. The others are divided into experimental groups, so that the male and female ratio is the same in each group and submitted to the following treatment schedule:

1, 2, 3, 4, 5, —, —, 8, —, 10, —, 12, —, —, 15, —, 17, —, 19 days.

On day 22 the animals are sacrificed and the weight of their tumours is determined. At the beginning and at the end of the treatment the body weight is recorded. Since during the treatment course some mice develop more than one tumour, evaluation of total weight of tumours per mouse is made. The data concerning the antitumour activity are expressed as inhibition of the average tumour weight of treated mice compared to that of control mice.

For statistical analysis the *t* Student's test is used.

C_{10} CANCER CELL DISSEMINATION AND METASTASIS

(1) *Cancer cell dissemination from tumours transplanted intracerebrally (i.c.) in Swiss Albino mice*

One hundred thousand cells of Sarcoma or Ehrlich carcinoma suspended in 10 µl of phosphate buffer pH 6·9 are injected i.c. in the thalamic area in the median line with a Hamilton microsyringe.

The presence of neoplastic cells disseminated in the blood and lungs of mice bearing i.c. tumours is evaluated by a bioassay procedure [14]. Detection of cancer cells in the lungs and blood begins to be positive on the 4th day after i.c. tumour transplantation, and progressively increases until animal's death (about the 14th day). Seven days after i.c. transplantation, treated and untreated mice (treatment beginning on the first day after transplantation) are killed by cutting the heart *in situ* in order to collect blood and lungs aseptically. Blood clot and lung fragments (about 40 mg) are then transplanted subcutaneously into normal recipient mice. Both tissues (blood clot and lung) of each donor are used for two subcutaneous transplantations. The number of tumour takes in recipient mice observed at the end of a 30-day period is a reliable parameter for the evaluation of different concentrations of cancer cells present in the transplanted tissue. The activity of the drug under test is expressed as a ratio between the percentage of tumour takes in recipient mice for each tissue from treated and untreated animals. For statistical analysis the χ^2 test is used.

(2) *Lymph node metastases from Ehrlich carcinoma transplanted intratibially*

One hundred thousand cells of Ehrlich carcinoma suspended in 10 µl of buffer phosphate are injected into the marrow cavity of the left tibia by means of a Hamilton microsyringe. Tumour growth results in macroscopic metastases are mainly located in the lymphatic system [15]. The metastatic lymph nodes are counted and weighed at autopsy after natural death. Microscopic sections are prepared when doubt exists about the nature of the nodule. The activity of the drug under test is expressed as a ratio between the percentage of animals with metastases and the number and weight of metastases, for treated (treatment begins 3 days after tumour implantation and is continued until the animals' death) and untreated animals.

For statistical analysis the χ^2 test and *t* Student's test are used.

(3) *Lung metastases from Lewis Carcinoma transplanted intramuscularly in C57B1/10J/Sel inbred mice*

An injection of 0·1 ml of tumour homogenate (1/50) in phosphate buffer pH 6·9 is given into the muscle of the left posterior leg of mice. Tumour growth results in metastatic nodules on the surface of the lung easily countable and measurable [16].

The activity of the drug under test is expressed as a ratio between the percentage of animals with metastases and the number and weight of metastases for treated (treatment begins 3 days after tumour implantation and is continued for 22 days) and untreated animals.

For statistical analysis the χ^2 test and *t* Student's test are used.

C_{11} HAEMATOLOGICAL TEST

(1) *Primary tests*

Substances which are considered active in one or both of the *in vivo* screening models (L 1210 or Ehrlich Ca) are tested in two doses (one single application). Both doses should have an antitumour effect.

Mouse strains: CBA; number in group: 5; application: orally, s.c., i.p., i.v. as in the above-mentioned tumour systems.

(*a*) Before treatment: peripheral blood smear, total leukocyte count with differential, erythrocytes, haemoglobin, reticulocytes, thrombocytes.

(*b*) 3 days after treatment: peripheral blood smear, total leukocyte count with differential, erythrocytes, haemoglobin, reticulocytes, bone marrow smear, spleen weight, thrombocytes.

(2) *Secondary tests*

If the substance tested shows pronounced effects either on tumour growth or on bone marrow behaviour (granulocytes or lymphocytes below 500/mm^3, reticulocytes below 10,000/mm^3), more detailed studies are suggested. Systematic studies should be done concerning the degenerative bone marrow phase (days 0 to 3), the phase of possible abortive regeneration (days 4 to 8) and the regenerative phase (until day 14). These studies should be done in close connection with the stem cell investigations (see C_1).

C_{12} FRIEND LEUKAEMIA VIRUS/MOUSE SYSTEM

The Friend leukaemia virus (FLV)/mouse system is chosen. The evaluation of the spleen weight of FLV infected treated mice and that of infected non-treated mice is used as the parameter of antiviral activity. This method was described by Sugiura [17] and is now used by other laboratories [18] as a method of *in vivo* screening of oncogenic virus activity.

Mice of the STU-inzest-mouse-strain at the age of 8 weeks and having a body weight of 20 g are infected intraperitoneally with FLV. For this test a virus concentration is chosen, which produces an enlargement of the spleen up to 1500 mg, after 21 days post-infection (p.i.).

The substance to be tested is injected intraperitoneally or subcutaneously into the mouse in an appropriate concentration either at the first day p.i. and/or at the seventh day p.i. All mice are sacrificed on the twenty-first day p.i. The average spleen weight of the infected and treated mice is compared with the average spleen weight of the infected non-treated mice. The activity of the substance is determined by the difference in spleen weights.

This *in vivo* test can also be quantitatively and qualitatively improved by administering the substance at various concentrations and in different modes of application.

C_{13} SPECTRUM OF ACTIVITY IN VARIOUS TUMOURS

(1) *Preliminary toxicity tests*

If unknown, compounds are investigated to determine the most appropriate solvent for administration, eg. H_2O, weakly acidic or alkaline solutions, aqueous propylene glycol, dimethyl sulphoxide and dimethyl-acetamide or arachis oil. Completely insoluble compounds are administered as a suspension in arachis oil. Unless otherwise specified, all tests are carried out by intraperitoneal injection of the test substance. The dosage schedules chosen are: one single administration for alkylating agents and related compounds, and five daily doses for all other materials.

The aim of the test is to provide, in one experiment, a therapeutic index which is some measure of the ratio of the tumour inhibitory or tumour curative dose to the LD_{50}. To select the dose range to be employed in the screening test which must range from lethal (to enable the calculation of the LD_{50}) to non-tumour effective (to enable the calculation of the tumour inhibitory dose) a preliminary toxicity test is carried out as described by Rosenoer *et al.* [19]. The basic features of this test are shown in Table 2. Normal animals (groups of 2) are injected with the compound under test at 625, 125, 25 and 5 mg/kg at the dose schedule, route of administration and solvent previously determined. From the numbers of animals that die at each dose level or undergo marked weight loss, the dose range is chosen for the full screening test by reference to Table 2.

(2) *Combined therapeutic toxicity test*

Groups of 3–6 animals are used in the screening test and, depending on the system used, treatment commences on day 1, 3 days after transplantation or when the tumours are palpable. Tumours are assigned at random to each group. Assay of anti-tumour effect is by measurement of tumour weight and comparison with a control or solvent control group, or by measurement of survival time and reference to a previously obtained cell inoculum-survival curve.

In some cases the rate of growth of the tumours of treated groups may be compared with the rate of growth of the tumour of a control group. The values for the tumour inhibitory dose and the LD_{50} are obtained graphically and enable the calculation of a therapeutic index [19].

Table 2. Selection of doses for therapeutic-toxicity tests

Results of preliminary toxicity test in 2 normal rats/dose (mg/kg i.p.)*				Doses chosen for therapeutic-toxicity tests (mg/kg i.p.)					
625	125	25	5						
0	0	0	0	1600	800	400	200	100	50
1 or +	0	0	0	1280	640	320	160	80	40
2	0	0	0	640	320	160	80	40	20
2	1 or +	0	0	320	160	80	40	20	10
2	2	0	0	160	80	40	20	10	5
2	2	1 or +	0	48	24	12	6	3	1·5
2	2	2	0	32	16	8	4	2	1
2	2	2	1 or +	9·6	4·8	2·4	1·2	0·6	0·3
2	2	2	2	6·4	3·2	1·6	0·8	0·4	0·2

*Numbers are the rats that died, + = marked weight loss, 0 = no effect.

Table 3

C_{13}	Tumour	Host	Time of injection	Assessment
(*a*)	Walker 256	Wistar rat	Day 1	Tumour weight
(*b*)	Walker 256 R*	Wistar rat	Day 1	Tumour weight
(*c*)	Walker 256 ascites	Wistar rat	Day 1	Survival time or cell number
(*d*)	Walker 256 ascites R*	Wistar rat	Day 1	Survival time or cell number
(*e*)	Yoshida Sarcoma	Wistar rat	Day 1 or 7	Tumour weight
(*f*)	Yoshida Sarcoma	Wistar rat	Day 1 or 7	Tumour weight
(*g*)	Yoshida Ascites	Wistar rat	Day 1	Survival time or cell number
(*h*)	Gardner Lymphoma	CBA/LAC mouse	Day 1 or palpable	Tumour weight or growth rate
(*i*)	Gardner Ascites	CBA/LAC mouse	Day 1	Survival time
(*l*)	Gardner Ascites Asp R†	CBA/LAC mouse	Day 1	Survival time
(*m*)	R_1 Lymphoma	CBA/LAC mouse	Day 3	Survival time
(*n*)	R_1 Lymphoma Asp R†	CBA/LAC mouse	Day 3	Survival time
(*o*)	NK Lymphoma	BALB/c̄ mouse	Day 1 or palpable	Tumour weight
(*p*)	PC5 Plasma Cell tumour	BALB/c̄ mouse	Day 1 or palpable	Tumour weight or growth rate
(*q*)	PC6 Plasma Cell tumour	BALB/c̄ mouse	Day 1 or palpable	Tumour weight or growth rate

*Lines developed with acquired resistance to various alkylating agents.
†Lines developed with acquired resistance to asparaginase.

(3) *Tumours available*

The tumours available and details of the test protocol are shown in Table 3. In addition to tests on tumour-bearing animals, a simple *in vitro—in vivo* system (C_{13}r) is also used for measuring the toxicity of small amounts of materials to these tumours. Washed ascites cells of the various tumours are suspended in TC 199/horse serum at a known cellular concentration and incubated with the drug under test for two hours at 37°C at a range of drug concentrations. After incubation the cells from the various incubations are injected into groups of recipient animals and their survival time is measured. Reference to the survival–cells–inoculum curve allows one to calculate the number of tumour cells killed by each concentration of the drug.

C_{14} CARCINOGEN-INDUCED, HORMONE-DEPENDENT MAMMARY CARCINOMA OF THE RAT

The test is used only for drugs interfering with hormone secretion or action.

Mammary tumours are induced in female Sprague–Dawley rats by a single feeding of 20 mg of 7,12-dimethylbenz(*a*)anthracene (DMBA) at the age of 50 days. Single or multiple tumours start to develop 6 weeks later and are present in nearly all rats within 3 months. These tumours are of interest as an

experimental model for the human female breast cancer because of their properties of hormone dependence.

They regress as a result of interference with secretion or activity of hormones such as oestrogens, prolactin or insulin. Drug evaluation is carried out as follows. About 10 weeks after DMBA, tumour-bearing rats are matched into "blocks" [20] according to number and size of tumours. The size of a tumour is measured by means of a caliper and expressed as "surface", that is, product of two perpendicular diameters. The rats are then randomized within matched blocks into treatment and control groups of 15 rats. The drugs are administered daily, 6 days per week, for 6 weeks. The change in total tumour "surface" per rat (as well as in body weight) is then recorded. Treatment effects are estimated by the differences in change of tumour surface between the matched rats. Statistical analysis is carried out by the Wilcoxon test on the paired differences.

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Influence of Local Environment on the Growth Parameters of the Ehrlich Ascites Tumor

P. K. LALA

Department of Anatomy, McGill University, Montreal, Quebec, Canada

Abstract—*Partial reduction in the mass of the advanced Ehrlich ascites tumor in mice led to a prompt rise in the growth fraction of the remaining tumor; this rise was only transiently delayed by a reintroduction of the cell-free fluid. Diffusion chamber implants of tumor cells into ascites tumors of different ages exhibited similar growth fraction and rate of DNA synthesis to those of the tumor cells surrounding the implants, indicating that these parameters were dictated by the local environment. During tumor growth, toxic products appeared to build up in the ascitic plasma; these products had a retarding effect on the DNA synthesis of bone marrow myelocytes* in vitro.

INTRODUCTION

A DECELERATION of the growth rate with increasing size or age of the Ehrlich ascites tumor has been found to result from a prolongation of the mitotic cell cycle and a decline in the growth fraction [1–3] in the tumor subline maintained in our laboratory. A prolongation of the cell cycle in the aging ascites tumor has been confirmed for a large number of mouse tumor lines [4–10], although growth fraction changes have been a less constant feature. These changes seen in the aging ascites tumor are reversible upon retransplantation of a fraction of the tumor into a fresh host [5, 8, 11, 12]. Since they are not associated with obvious changes intrinsic to the tumor cells, such as gross changes in chromosomal constitution [8; Lala, unpublished data], they presumably result from changes in the environment. Environmental alteration may, in turn, be of a general and non-specific nature pertaining to the tumor bearing host or of a specific one, taking place within the tumor. A gradual deterioration of the nutritional status of the host illustrates the former case. However, we have failed to detect any change in the cell cycle time or the growth fraction of bone marrow cells of mice bearing advanced ascites tumor [13]. This would suggest that an alteration of these growth parameters in the aging ascites tumor does not reflect non-specific changes taking place in other host tissues as well; it must result from environmental changes within the tumor. Although studies have been made on the quality of the environment in the aging ascites tumor [14], or its effects on the rate of tumor growth [15], the precise relationship between the environmental factors and the individual growth parameters such as the growth fraction and the cell cycle time has not been firmly established. Information combined from a large number of studies has clarified the picture substantially [3, 5–9, 11–13, 16–22], and the present study was aimed at further elucidating this relationship in the Ehrlich ascites tumor.

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MATERIAL AND METHODS

Ehrlich ascites tumor employed in the present study is a subline having a modal chromosome number of 68 and near-tetraploid mode of DNA-content in the post-mitotic cells. It is routinely maintained by a weekly intraperitoneal transfer of 10^6 tumor cells from 7-day old tumors, into 10–12-week old CF_1 female

mice weighing about 25 g. Tumor bearing animals survive for a period of approximately 2 weeks. Growth curves of this tumor have been published [1, 23]. Four-day and/or 7-day old tumors were used in the present study depending on the experiment. Animals of similar weight were chosen from a tumor age in each experiment, since animals with identical weight showed fairly identical ascites fluid volume and tumor cell population size. Four-day old tumors had 2×10^8 cells in a 2 ml vol, and the 7-day old tumors had $6–7 \times 10^8$ cells in a 7–8 ml vol. All the surgical procedures were conducted under light general anesthesia (Diabutal 40 μg/g body wt subcutaneously, aided by light ether inhalation).

Ascites aspiration experiments

Effects of ascites aspiration on the growth parameters were studied in the 7-day old tumors. Aspiration was carried out with a fine heparinized needle. To prevent leakage, the site of peritoneal puncture was kept about 1/2 in. apart from the skin puncture site and, in addition, the needle track was closed by a subcutaneous silk suture. Approximately 5 ml fluid (4·5–5·3 ml) was removed in the experimental animals; in some, the cell-free fluid (supernatant after spinning at 4000 rev/min for 5 min) was injected back through the same needle kept *in situ*. The whole aspirate was injected back into the animals serving as sham-aspirated controls. Animals showing any subsequent leakage were discarded from the study. A single dose of H^3-thymidine (H^3TdR, 1 μCi/g, 6 Ci/mM, New England Nuclear Corp.) was injected intraperitoneally at varying intervals up to 48 hr following aspiration and animals were sacrificed 1 hr later for an estimation of the labeling index in the tumor smears.

Diffusion chamber experiments

Effects of the cell-free environment *in vivo* on the tumor growth parameters were evaluated in these experiments. 0·1 ml samples of ascites fluid containing the free tumor cells were removed through fine, heparinized needles from 4-day and 7-day old tumors. These were placed in millipore diffusion chambers (i.d. = 10 mm; thickness = 2 mm, pore size = 0·45 μ), which were then sealed and surgically implanted into ascites tumors of various ages. Experimental groups constituted a two-way cross-implantation; 4-day old tumor→7-day old tumor, and 7-day old tumor→4-day old tumor. Control groups were provided by the auto-implants of 4-day or 7-day old tumor cells inserted back into the donor tumors. Wounds were kept as small as possible to facilitate leakage-free closure. Peritoneum and muscle layers were closed with continuous silk sutures, and the skin with wound clips. Despite all the care, a leakage-free operation succeeded only in about half of the 7-day old tumors; those showing leakage were discarded from the analysis. H^3TdR (1 μCi/g, 6 Ci/mM) was injected intraperitoneally into all the animals 18 hr after implantation of the chambers. Animals were killed 1 hr later and tumor cells collected both from within as well as outside the implanted chambers were smeared on slides for radioautography. For a determination of the labeling indices of tumor cells prior to implantation into a tumor of a different age, H^3TdR was injected into the donor tumors immediately following aspiration, and the tumors were gathered 1 hr later for making smears before radioautography.

In vitro *experiments*

The effects of cell-free fluid from the ascites tumors on the short term growth of mouse bone marrow cells were evaluated in these experiments. Bone marrow cells from both the femurs were collected from 5 mice of the same strain and sex as that of the tumor bearers, and pooled into 5 ml Hank's balanced salt solution which was filtered through a millipore swinny filter holder, containing the steel sieve but no filter, to get rid of blood vessels. The cell suspension was divided into ten 0·5 ml aliquots, each containing 5×10^6 nucleated cells, and was cultivated with 2 ml Eagle's minimal essential medium containing varying concentrations of fresh blood plasma or ascitic plasma: I, 20% homologous blood plasma from normal mice; II, 20% ascitic plasma from 3-day old tumor; III, 10% ascitic plasma from 9-day old tumor, IV, 20% ascitic plasma from 9-day old tumor; and V, 40% ascitic plasma from 9-day old tumor. For cultures III, IV and V, plasma from the same ascites tumor was used. Duplicate cultures for each of the above types were incubated at 37°C for 3 hr. At the end of the incubation period H^3TdR (1 μCi/ml, 6 Ci/mM prewarmed to 37°C) was added to the cultures, and incubation was allowed to continue for another 20 min. Cells were then directly fixed in suspension by adding 10 ml freshly prepared fixative (acetic acid : methanol, 1 : 3 by vol) to each culture. At the end of 10 min, cells were spun and suspended in 10 ml fresh fixative; this was repeated twice and the centrifuged cell button was uniformly suspended in several drops of fixative. One drop of the

suspension was then allowed to spread on an obliquely held, precleaned slide moistened in ice-cold water. Slides prepared as above were dried on a hot plate and dipped for 15 min in absolute methanol before storage for radioautography. Cellular morphology was well retained in these preparations, which could be stored indefinitely.

Radioautography and evaluation of radioautograms

Smears fixed for 40 min in methanol, as well as the spreads of bone marrow cells were dipped in NTB–2 nuclear emulsion (Eastman Kodak) diluted 1 : 1 by vol with distilled water at 45°C. They were developed after 3 weeks' exposure in the dark, and stained with Giemsa. All the slides from a single experiment were handled in one batch in an identical way. On the basis of grain count distribution of background and cells with overlying silver grains [1], the threshold for a positive labeling was set at 5 grains for the tumor cells, and 4 grains for the bone marrow myelocytes. A minimum of 2000 tumor cells were taken for a determination of labeling indices, and 500 labeled tumor cells were taken for grain counts. Myelocytes alone (including the early ring forms) were scored in the bone marrow preparations; the minimum score was 500 cells for labeling indices, and 200 labeled cells for the grain counts.

RESULTS

The cell cycle time and the growth fraction in this type of tumor are variable parameters depending on the total tumor mass—which, in turn, depends on the inoculum size and the age of the tumor [3]. Nevertheless, the duration of DNA synthesis occupies a relatively constant proportion of the cell cycle irrespective of the stage of growth [1, 3, 23]. This finding permits an estimation of growth fraction directly from the measurement of the pulse H^3TdR-labeling index, since growth fraction is computed as the ratio of the observed fraction in DNA synthesis to the expected fraction, if all the cells were in cycle [1, 16]. The expected fraction can be computed from the knowledge of the cell cycle components [16]. Figure 1 represents the relationship between the H^3TdR-labeling index and the growth fraction in this ascites tumor. Data were derived from eight different measurements of the tumor cell cycle at different stages of growth including both published [3, 23] and unpublished materials. The linear relationship suggests that changes in growth fraction can

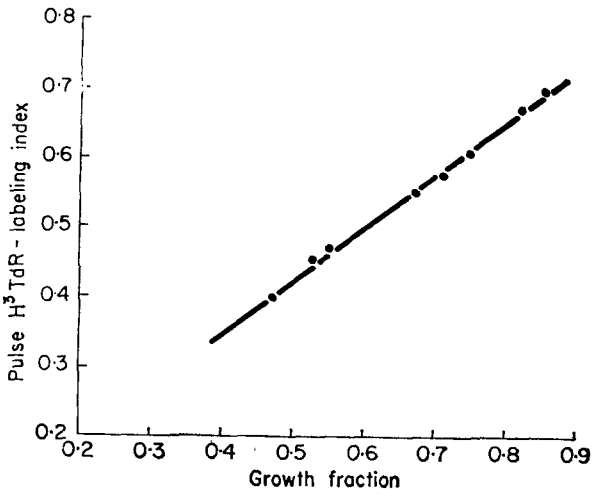

Fig. 1. Relationship between the observed fraction of the cell population synthesizing DNA and the growth fraction in the Ehrlich ascites tumor.

be evaluated directly from changes in the pulse H^3TdR-labeling index in this tumor.

Results of the ascites aspiration experiment are indicated in Table 1 and Fig. 2. Aspiration of approximately 5 ml ascites (out of a total 7–8 ml) in the 7-day old tumor resulted in a rapid rise of the H^3TdR-labeling index. The peak was attained within 12 to 18 hr followed by a decline parallel to that shown by the unaspirated control animals. If the aspirated tumors, after homeostasis had taken place, behaved as undisturbed tumors of similar mass, then they should correspond kinetically to 4 to 5 day old tumors. A correlation between the peak labeling index in the aspirated tumors and the labeling index at 4·5 days of normal tumor growth supports this hypothesis. Sham-aspirated controls in which the aspirated material was reintroduced, behaved similarly to the unaspirated controls. Although the rise in the labeling index was apparently slightly delayed by a replenishment of the cell-free fluid into the aspirated animals, the overall labeling pattern did not seem to be affected by this procedure. The excess fluid was possibly eliminated from the peritoneal cavity within a period of 6 hr or so, as indicated by the initial weight loss in these animals; following this, their weights were similar to those of the aspirated animals in which the cell-free fluid was not replaced.

Results of the diffusion chamber experiments are presented in Figs. 3, 4 and 5. Figure 3 compares the 1 hr H^3TdR-labeling indices of the tumors donating cells for implantation and the indices within the grafts 18 hr after implantation into tumors of different ages. This time was considered adequate for an environ-

Table 1. Percentage of tumor cells in DNA synthesis following ascites aspiration in the 7-day old tumors, and in the unaspirated tumors of comparable age

Interval hr	Mean H^3TdR labeling index (percentage, ± 1 standard error)			
	Unaspirated	Sham-aspirated	Aspirated	Aspirated and cell-free fluid replaced
0	43·0 ± 2·0	—		—
4	—	—	44·7 ± 2·0	—
6	—	—	—	44·0 ± 2·1
12	—	40·7 ± 2·3	53·6 ± 2·5	—
13·5	—	—	—	50·5 ± 2·5
18	—	—	53·9 ± 2·4	—
24	—	40·1 ± 2·6	52·0 ± 3·0	—
26	39·5 ± 2·0	—	—	52·5 ± 2·9
45	—	—	—	47·6 ± 2·5
48	—	38·0 ± 2·8	48·6 ± 2·4	—
50	36·0 ± 2·1	—	—	—

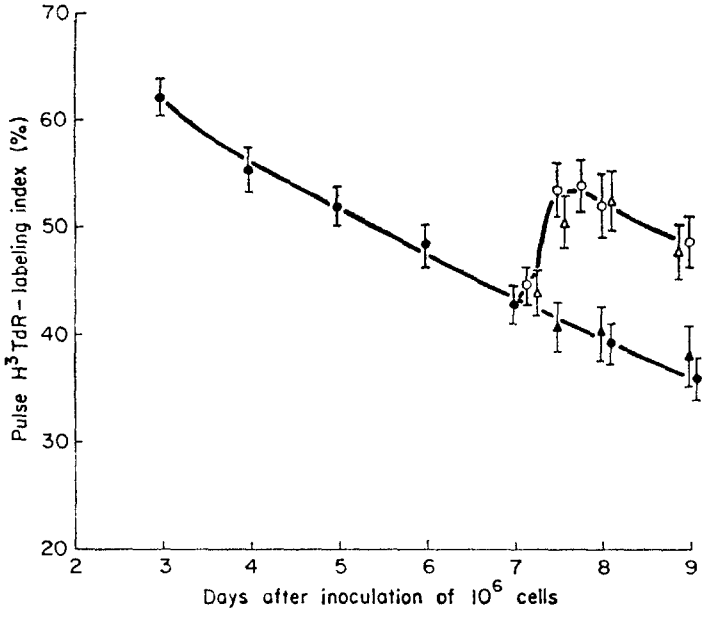

Fig. 2. Pulse H^3TdR-labeling indices during undisturbed ascites tumor growth, compared to the labeling indices following aspiration in the 7-day old tumor. Individual points represent the mean of 4 tumors (± 1 standard error). ● *Control undisturbed tumors;* ▲ *sham-aspirated controls in which the total aspirate was reintroduced;* ○ *aspirated tumors;* △ *aspirated tumors in which the cell-free fluid was reintroduced.*

mental homeostasis between the cells within and outside the diffusion chambers. Cells from the 7-day old tumors showed a marked rise in the labeling index when placed in the environment of a 4-day old tumor. A reverse procedure led to a substantial decline in the labeling index. Final values attained within the grafts were comparable to values shown by tumors of similar ages as those of the host tumors (Fig. 2) and very close to the values shown by the host tumors themselves (Fig. 4). Nevertheless, irrespective of the direction of the implant, the labeling index within the implant was always slightly lower than that of the tumor nursing the implant (Fig. 4), suggesting that the cells within the chambers may have suffered from

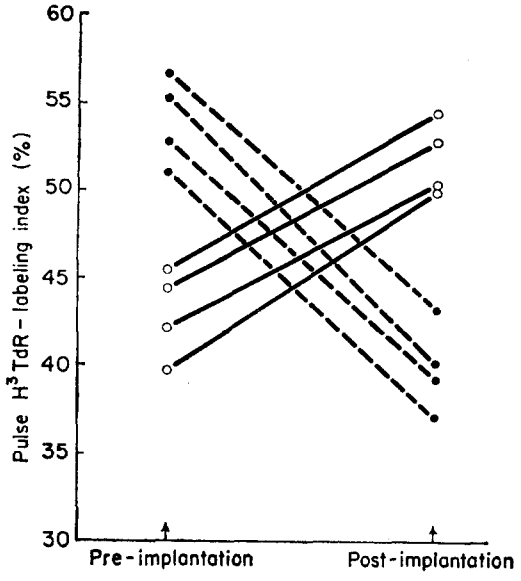

Fig. 3. Pulse H^3TdR-labeling indices of tumor cells prior to and 18 hr after implantation into an ascites tumor of different age. ○——○ *7-day→4-day;* ●——● *4-day→7-day*

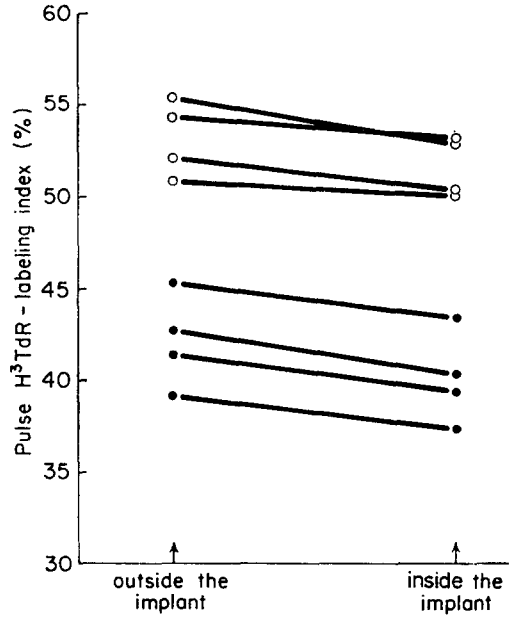

Fig. 4. Pulse H^3TdR-labeling indices of the implants compared to that of the different aged tumors nursing the implants. ○——○ *7-day→4-day;* ●——● *4-day→7-day.*

some kind of handicap in labeling. This suggestion is reinforced by identical findings, even in the auto-implants (Fig. 5) serving as controls. The minor, but consistently observed labeling handicap of cells within the diffusion chambers may be explained either by a poorer thymidine penetration, or by a relative deprivation of biological factors dictating the growth fraction. The former can be largely excluded from the findings on the median grain counts

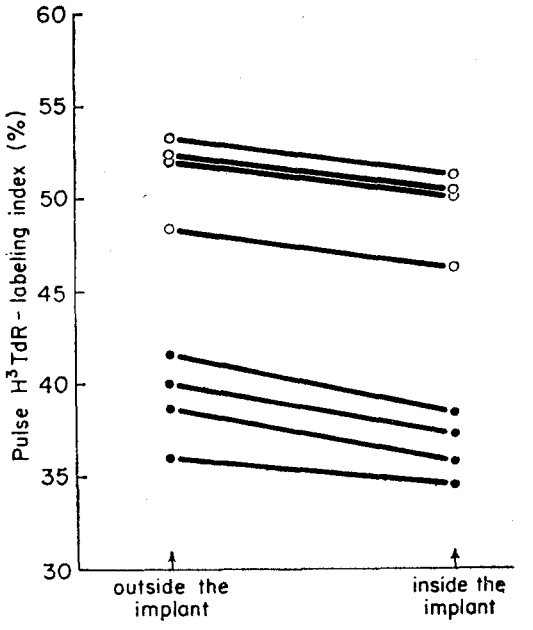

Fig. 5. Pulse H^3TdR-labeling indices of the implants grafted back into the donor tumor, compared to those of the cells outside the implants. ○——○ *4-day tumors;* ●——● *7-day tumors.*

of labeled cells inside and outside the implants in the various forms of implantation procedures (Table 2). Irrespective of the direction of implantation, the counts were very similar in cells inside and outside the diffusion chambers, indicating an identical rate of incorporation of the label.

The results obtained from the *in vitro* experiments with bone marrow cells are

Table 2. Median grain counts of labeled cells outside and inside the implants in the various forms of implantation

Direction of implantation	Animal number	Outside the implant	Inside the implant
4-day→7-day	1	21	20
	2	25	25
	3	24	23
	4	22	21
7-day→4-day	1	33	32
	2	36	36
	3	29	28
	4	30	30
4-day→4-day (auto-implant)	1	32	31
	2	36	35
	3	30	30
	4	37	36
7-day—7-day (auto-implant)	1	26	24
	2	23	22
	3	20	20
	4	21	20

summarized in Table 3. There was a small and gradual drop in the labeling index of myelocytes from cultures I–V. The median grain count showed a more marked decline in

*Table 3. Results of H^3TdR-labeling of bone marrow myelocytes following cultivation in duplicate with blood plasma and ascitic plasma**

		I	II	III	IV	V
Labeling index	A	43·6	42·2	40·6	39·0	36·2
	B	42·2	41·2	41·0	40·4	33·8
Median grain count	A	23	20	16	14	12
	B	22	19	17	14	10

*For a composition of the five sets of cultures see "Material and Methods".

the same order. Comparison of cultures I (20% normal blood plasma) with cultures II (20% 3-day old ascitic plasma) and culture IV (20% 9-day old ascitic plasma) indicates that normal blood plasma was superior to 3-day old ascitic plasma, which was, in turn, superior to 9-day ascitic plasma in supporting the DNA synthesis of bone marrow cells *in vitro*. This could result from an increasing deficiency or toxicity, or from both. A comparison of the effects of increasing concentrations of 9-day ascitic plasma (cultures III, IV and V) indicates the presence of toxic factors. Although these results do not exclude the presence of molecules competing with labeled thymidine in the tumor plasma, the presence of inhibitors retarding the rate of DNA synthesis appeared to be a more likely explanation since cytotoxic factors having similar [21, 24] or different [25, 26] biological effects have been identified in the ascites tumor fluid.

DISCUSSION

Present studies directly demonstrate that the characteristic alteration in the growth rate seen in the aging ascites tumor is dictated by changes in the local environment.

Sustained growth of ascites tumor cells has been demonstrated in diffusion chambers inserted in the peritoneal cavity of normal mice [27, 28]. Present experiments, in which chambers were grafted into an ascites tumor, showed that both the H^3TdR-labeling index as well as the median grain count in the labeled cells within the chambers were dictated by the environment of the nursing tumor. This suggests that both the growth fraction and the rate of DNA-synthesis (consequently the duration of DNA-synthesis) were controlled by the local environment in the ascites tumor. Since DNA synthesis occupied a proportionate interval in the cell cycle at all stages of tumor growth [3, 23] the same conclusion also applies for the total cell cycle time.

The minor handicap suffered by the implants resulting in a small drop in the growth fraction can best be explained by a poorer availability of oxygen. Cells outside the implants were free to move, aided by the peristaltic movements of the intestine and had a better probability of coming into proximity with the peritoneal capillaries, compared to the cells within the diffusion chambers. Distance from the capillary appears to be a crucial factor dictating growth fraction in the solid tumors [18] by determining the extent of oxygenation. Hypoxia can cause a decline in the growth fraction without any appreciable alteration of the cell cycle time both in the solid [19] and in the ascites [20] tumor.

A rise in the labeling indices caused by ascites aspiration could theoretically result from a preferential shortening of $G_2 + \text{mitosis} + G_1$ relative to the DNA synthesis time in the aspirated tumor, or a true increase in the growth fraction due to the entry of non-cycling cells into cell cycle. Since we found no evidence for the former while ascites cells were rejuvinated by transplantation into a new host [11], it may be concluded that changes in labeling indices would reflect true growth fraction changes. An increase in growth fraction of the remaining tumor in the aspirated animals could result from (*a*) an increase in the availability of nutrients, including oxygen, per cell, or (*b*) partial removal of toxic products, or (*c*) partial removal of some form of contact inhibition. Since the number of cells/ml of ascites fluid remains fairly stable during the major part of tumor growth [1] while drastic changes in the growth rate continue, contact inhibition resulting from crowding may largely be excluded in this tumor. Present experiments largely favor the idea of a partial correction of deficiency, rather than toxicity, improving the growth fraction in the aspirated tumor, since the labeling pattern did not substantially change following the re-introduction of the cell-free fluid. However, since the animals were apparently able to eliminate the excess fluid within a short time, it is possible that the excess toxic products in the fluid were eliminated as well. This may explain the differences in the present findings from those of Bichel [15], who found a significant suppression of the mitotic index by injecting the cell-free fluid

twice daily into the aspirated tumor. In a later study [22], Bichel also found that the growth-retarding effect of a single injection of the cell-free fluid was rather short-lived.

Present results of ascites aspiration, as well as qualitatively similar results reported by others [8, 12, 15, 22], are consistent with our previous findings that the growth rate of the ascites tumor depended more on the total tumor mass than tumor age; the initial growth rate, the cell cycle time and the growth fraction were all influenced by an alteration of the inoculum size [3]. This would suggest that the extent of availability of nutrients/cell influences both the cell cycle time and the growth fraction within this tumor.

A drastic decline in the median grain counts and a small drop in the labeling index of myelocytes observed in the *in vitro* experiment, are best explained by a retardation of the DNA synthesis rate caused by toxic products in the ascitic plasma. Although the DNA synthesis time and the cell cycle time are possibly influenced by both deficiency and toxicity, the latter must have taken an upper hand in the present case. In our previous studies we found no change in the DNA synthesis time (or the cell cycle time) of myeloid–erythroid precursor cells in the marrow of animals bearing advanced ascites tumor [13]. This can be explained in two ways: (*a*) inhibitors from the tumor circulated in the blood of the host, but did not affect the proliferation of bone marrow cells, possibly because they were tissue-specific—conforming to the definition of Chalones [15, 22] or (*b*) inhibitors were non-specific, but did not circulate in a concentration large enough to affect proliferation of host tissues. The latter view is supported by the present *in vitro* findings, as well as those of others [24–26]. Nevertheless, this study does not exclude the possibility that inhibitors of both kinds, specific as well as non-specific, may be present in the ascitic plasma. The latter substances are possibly products of cell lysis. If they affect the cell cycle time more than the growth fraction, one may explain why the cell cycle does not change drastically within the solid tumor [17, 18, 29, 30] in comparison to the aging ascites tumor. The toxic factors diluted in the ascitic plasma would then affect the cell cycle of all the ascites tumor cells, whereas increased local concentration of such factors would lead to cumulative death of cells with retarded cycle in the poorly nourished zones of a solid tumor. This suggestion is an alternative to the hypothesis proposed by Tannock [7], which postulates that anoxia is the prime reason for the cell cycle prolongation in any tumor; the difference between the solid and the ascites tumor is believed to result from the higher survival value of the long cycling cells in the ascites tumor, attained by (*a*) intermittent oxygenation, and (*b*) a dilution of toxic factors.

Increasing deficiency and toxicity both cause the deterioration of the quality of the environment within the growing ascites tumor. The following question naturally arises: do both these factors equally affect the two growth parameters, viz. the growth fraction and the cell cycle time? Although no conclusions can be drawn from the present study, some suggestions may be made. It may be tentatively suggested that growth fraction is more sensitive to deficiency (of oxygen in particular) than to toxicity. Recycling process, through the initiation of DNA synthesis, being more energy-dependent than any other process in the cell cycle, may suffer more from deprivation [20] than toxicity. The cell cycle time is influenced by both, but possibly more so by toxicity. In addition, toxic factors also possibly limit the life span of the most deprived cells, viz. the non-cycling cells [23] as well as cells with extremely long cycles, which cannot be kinetically distinguished anyway.

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Clinical Trials with Bleomycin in Lymphomas and in Solid Tumors

G. BONADONNA, M. DE LENA, S. MONFARDINI, C. BARTOLI, E. BAJETTA, G. BERETTA and F. FOSSATI-BELLANI

National Cancer Institute, Milan, Italy

Abstract—*The authors report the results of Phase I and Phase II evaluation of intravenous bleomycin (BLM) in 176 patients. During the trial different doses and schedules were employed. The less toxic regimen was that of 15 mg/m² × 2/week per 4 weeks. Irrespective of the schedules, the side effects are represented by sclerotic changes of the skin of hands (88%), skin hyperpigmentation (78%), fever (70%), loss of hair (68%), stomatitis (47%) pulmonary toxicity (42%), gastro-intestinal symptoms (20%). No significant myelo-suppression was observed. Pulmonary toxicity occurred after 4–10 weeks from starting therapy and was detected on clinical bases (fine crepitations at the base), radiologically (reticulonodularity mostly at the lower lung zones) and histologically (edema of alveoli, hyaline membranes formation, collagen deposition). Upon prompt discontinuation of BLM, the signs of pulmonary damage remained unchanged or regressed in the majority of cases.*

Lower total doses of BLM produced a lesser incidence of lung toxicity. However, in about 5% of cases the presumptive cause of death could be related to extensive pulmonary drug-induced lesions. The incidence of lung toxicity was not found to be significantly related to the age of patients, to the total dose of BLM or to the presence of chronic pulmonary disease. BLM produced significant regression in all types of malignant lymphomas (40%), in epidermoid carcinomas of the head and neck (57%) and of the esophagus (60%). No significant responses were observed in epidermoid carcinomas of the lung. Regressions were prompt but usually short-lived with or without maintenance therapy. Because of its toxic and therapeutic properties BLM appears suitable for therapeutic trials either in short courses alone in or in combination with myelosuppressive agents.

INTRODUCTION

BLEOMYCIN (BLM) is a new antitumor antibiotic isolated in Japan by Umezawa [1] from a strain of Streptomyces verticillus. Its chemical structure is still under investigation. BLM is known to be a complex which can be fractionated by paper chromatography into 13 peptides. Two groups of peptides have been recognized: group A, (A_1, A_2, A_3, A_4, A_5, A_6 and A_7) and group B (B_1, B_2, B_3, B_4, B_5 and B_6). The compound for clinical use is copper-free and contains not less than 50% of A_2, not more than 3% of B_4 and not more than 35% of all B group substances [2].

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Correspondence and requests for reprints to Dr. Gianni Bonadonna, 1 Via Venezian, 20133-Milan, Italy.

The drug has been found to be active on some experimental mouse and rat tumors with life-prolonging effect. However, only a very moderate antitumor effect was observed in mouse L 1210 leukemia [3]. BLM inhibits the incorporation of thymidine into DNA of HeLa cells and *E. coli*, but does not inhibit the incorporation of uridine into RNA and that of amino acids into protein [4, 5]. Bleomycin A_2 reduced the melting temperature (MT) of DNA in presence of hydrogen peroxide or of a SH-compound. The drug complexes with DNA and uncoils its double helix [6, 7]. From 17 to 76% of BLM is inactivated when mixed and incubated with various tissue homogenates. Furthermore, SH-compounds inactivate BLM in presence of phosphate ions.

This has been thought to be due to absorp-

tion and linkage between BLM and chemical substances present in the tissues [8].

In experimental animals and in man, BLM is excreted in urine as such. In man, about 40% of the administered dose is excreted within 24 hr [8, 9] while this level was found to be higher in animals. In cancer patients, after a single i.v. injection of 15 mg, the maximum blood level averaged 3·3 mcg/ml and the half-life 14 min. There was a rapid fall during the first 30 min, but slowly thereafter. In patients receiving an equal i.m. dose, the maximum blood level averaged 0·97 mcg/ml. However, moderately high levels persisted for several hours [8]. The drug is distributed in high concentration in the skin, lungs, peritoneum, lymphoid tissue and epidermoid tumors [3, 8].

The acute LD_{50} in mice was 167 to 312 mg/kg and 143 to 226 mg/kg in rats. In subacute and chronic toxicity studies carried out in Wistar rats, the side effects were mostly represented by cachexia, loss of hair and of nails, thickening of the soles, hyperplasia of the connective tissue surrounding liver and spleen, congestion of lungs with thickening of the alveolar septa and appearance of foam cells without clear-cut fibrosis. No definite biochemical or hematological toxicity was detected.

This was interpreted by Shimada as lack of inhibition of dehydrogenase activity of human marrow cells by BLM [10].

In man, intravenous or intramuscular administration of BLM (15–30 mg twice weekly) produced a number of side effects [11–14]: pirexia (3·16%), stomatitis (6%), alopecia (27·7%), thickening of the skin of palms and soles (26·6 and 22·8%), skin hyperpigmentation (18·3%), distrophic changes of nails (13·3%), bronchopneumonia (4·2%) and pulmonary fibrosis (3·4%). In the initial Japanese series (468 patients) the mortality rate related to BLM pulmonary toxicity was reported to be 1·5% [11]. Pulmonary damage seems to occur after total doses above 200 mg, in elderly patients and more frequently if chronic pulmonary diseases are present. In the E.O.R.T.C. series, where BLM was administered at the dose of 10 to 20 mg/m^2/day × 10 to 38 consecutive days, 8 patients (3·3%) showed pulmonary toxicity and 5 of them (2·1%) died [15]. Unfortunately, both series lack details as far as the total toxic dose per patient, the time of onset of pulmonary symptoms, the radiological picture, the clinical course and the histopathologic findings are concerned. However, in 3 small series [16–18] Japanese investigators reported a higher incidence of pulmonary toxicity. The dose schedule of BLM was again 15 to 30 mg twice a week. Suzuki *et al.* [16] observed pulmonary damage in 12/24 (50%) cases with carcinoma of the esophagus after a total dose of 120 to 540 mg. Tamura *et al.* [17] detected at post mortem examination lung toxicity in 5/6 patients (83%) after a total dose of 150–375 mg. These authors provided a detailed description of pulmonary lesions (edema of alveoli and interstitial tissues, hyaline membrane formation, swelling of alveolar lining cells, increase of reticulin fibres of the alveolar wall and interstitial tissues, edematous thickening of the walls of small arteries and veins). They found also renal lesions in 5/6 cases (formation of hyaline trombi of the glomerular capillaries and of the afferent arteries with occasional necrosis of the capillary loops and severe hemorrhages in the Bowman's capsule). Araü *et al.* [18] observed pulmonary toxicity in 3/7 cases (42%). The histopathologic findings of one patient who died because of severe respiratory insufficiency are reported.

After the first clinical trials carried out in Japan, BLM was reported to show a specific therapeutic activity against all types of epidermoid carcinomas [11–14, 19). The most responsive form of tumor was carcinoma of the penis (81%), but also in carcinomas of the skin (61–75%), head and neck (68·2%), esophagus (75%), lung (68%), vulva and cervix (28%), BLM revealed a remarkable degree of efficacy. The drug proved also to be very active against malignant lymphomas (71·2%) and psoriasis. Most of these data (mainly responses) were confirmed by the E.O.R.T.C. group [15], where BLM was also reported to be moderately active against malignant melanoma, choriocarcinoma of the testicle, and a miscellaneous group of solid tumors (breast, kidney, ovary, intestine, etc.). The drug was administered in Japan and in Europe through the intravenous, intramuscular, intraperitoneal and intra-arterial routes. The duration of response with or without maintenance treatment has not been specified [11–13, 19].

More recently, the toxic and therapeutic properties of BLM were evaluated also in Italy [20, 21] and in U.S.A. [22–25]. The aim of the present study is to report the experience achieved in our Institute with different dose-schedules of BLM in malignant lymphomas and in various types of solid tumors. Part of these results has been recently presented at the annual meeting of the American Association for Cancer Research, held in Chicago [21].

MATERIAL AND METHODS

Patients and evaluation procedures

Of the 176 patients entered on study, (142 males and 34 females), 154 are suitable for the evaluation of acute and delayed toxicity, while only 147 may be evaluated to detect the therapeutic effect of the drug. On 147 patients, 146 had various types of neoplastic disease and 1 had psoriasis, Furthermore, 2 patients had a double tumor (cancer of the esophagus and cancer of the head and neck) while psoriasis was associated with the malignant lymphoma in 2 cases (Hodgkin's disease and lymphoreticular sarcoma). The average age was 54 years (minimum 7 and maximum 83). Twenty-five patients were untreated and 122 had received prior to BLM administration one or more courses of radiotherapy and/or chemotherapy. Thirty-four cases had been previously treated by surgery.

Liver function tests, blood urea nitrogen, fasting blood sugar, serum electrophoresis, serum electrolytes, serum uric acid, complete blood count and chest X-ray were obtained in all patients before drug administration. Bone marrow aspiration from the sternum or from the posterior iliac crest was carried out only in patients with malignant lymphomas, acute leukosarcoma and chronic lymphocytic leukemia. During the period of treatment and observation, chest X-ray was performed every 12 to 15 days and occasionally more often when pulmonary toxicity was evident or suspicious. Blood counts and analyses were repeated periodically and bone marrow aspiration every 8 to 12 days in patients with leukosarcoma or leukemia. Pulmonary function tests were not carried out during this study. The consent for post mortem examination was obtained in 11/15 cases who died in the hospital.

Drug and mode of administration

Bleomycin* hydrochloride is available as a whitish dry powder in 15 mg ampules. The compound is freely soluble in 0·9 % NaCl solution, distilled water or 5 % glucose in water. During this study BLM was diluted in 0·9 % NaCl, and the compound administered through rapid i.v. injection. Different doses and schedules were employed, as summarized in Table 1. The patients were randomly allocated to the first 2-dose schedules with the intent to compare an alternate vs. an intermittent loading dose-schedule. After evaluation of the incidence of the side effects observed with schedules A and B, a new 8-day schedule (C) was designed, in which the dose was reduced by 50 %. Since schedule C did not appreciably reduce the incidence of side effects and since acute toxicity appeared in the first 3 schedules between the 4th and 6th doses, BLM was then administered for 5 consecutive days (schedule D). Finally, to further reduce the incidence of delayed toxicity, a fifth schedule (E) was tried in some patients where BLM was injected twice a week, i.e. with a sequence similar to that originally employed by Japanese and E.O.R.T.C. investigators.

*Kindly supplied by Laboratorio Roger Bellon, Sesto San Giovanni, Italy.

Table 1. Doses, schedules and evaluable cases

Dose schedule (mg/m)²	Patients entered on study	Appraisable cases for toxicity	Appraisable cases for therapy	Description	Total dose (mg/m²)
A 30	25	18	16	BLM twice a week × 4 weeks. Course repeated after 1 month interval	480 in 12 weeks
B 30	20	18	17	BLM for 8 consecutive days. Course repeated after 1 month interval	480 in 8 weeks
C 15	33	31	29	BLM for 8 consecutive days. Course repeated after 1 month interval	240 in 8 weeks
D 15	81	70	68	BLM for 5 consecutive days. Course repeated twice at 3 week intervals	225 in 11 weeks
E 15	17	17	17	BLM twice a week × 4 weeks. Course repeated after 1 month interval	240 in 12 weeks
Total	176	154	147		

Maintenance treatment, always with the initial dose/m^2, was given once a week in the large majority of patients until either unequivocal signs of progression or definite lung toxicity were evident. Once pulmonary toxicity was detected, BLM was definitely stopped and corticosteroids (prednisone 50 mg/day) plus antibiotics were administered for 10 to 15 days. To prevent or minimize the febrile reaction, a suppository of indomethacin (100 mg) was given about 1 hr before injection.

Adequacy of treatment. During all this Phase-I and II study, an adequate dose of BLM has been defined: that producing one or more evident signs of toxicity. Table 2 shows that in the first 3 schedules only about half of the patients did complete the second course as originally planned (indicated in the table as A_2, B_2 and C_2). In fact, due to the increasing discovery of lung toxicity, we decided not to expose the patients to a higher dose of BLM within a relatively short period of time. Even with schedule D, which was expected to be less toxic, a more cautious attitude was taken during the study. Therefore, only 8 cases were actually given 3 courses as originally planned (indicated as D_3) while 31 received two courses (D_2) and 31 only one course (D_1) before maintenance therapy. With schedule E, on the contrary, to minimize the incidence of delayed toxicity, BLM was stopped for 3 to 4 weeks at the appearance of the first side effect (e.g. hyperpigmentation, sclerosis of the skin, stomatitis, or loss of hair).

Table 2. Number of courses in relation to the dose schedules and minimal toxic dose for the first five schedules

Dose schedule	No. cases evaluable for toxicity	No. courses	No. cases	Mean minimal toxic dose (mg/m^2)
A	18	A_2	8	146
		A_1	10	
B	18	B_2	8	200
		B_1	10	
C	31	C_2	15	87
		C_1	16	
D	70	D_3	8	
		D_2	31	70
		D_1	31	
E	17	E_1	17	65

The patients were considered suitable for the evaluation of acute and delayed toxicity if they were observed for at least 1 month after the last dose of BLM. The therapeutic effects of BLM have been classified according to the categories of response proposed by Karnofsky,* which were reprinted in a previous issue of this Journal [26]. The duration of response was measured from the time of initial but unequivocal tumor regression to the time of objective evidence of progression or relapse.

RESULTS

Toxicity

The incidence of all toxic manifestations recorded at the end of the drug course irrespective of the total dose and the schedule is reported in Fig. 1. Sclerotic changes of the skin of hands (less frequently of feet) with edema of the fingers and occasionally with distrophic changes of the nails (Fig. 2) represented the most frequent side effect. Furthermore, in about half of the patients this occurred also as the first sign of toxicity. Skin hyperpigmentation, mostly in the form of hyperpigmented stria located throughout the back (Fig. 3) comes next, followed by loss of hair, stomatitis, pulmonary toxicity, gastro-intestinal symptoms (nausea, vomiting and mild diarrhea) and diffuse allergic-like erythema. While stomatitis regress occurs within 8–12 days, complete regrowth of hair takes about 3–4 months. Skin hyperpigmentation, on the contrary, did not show any tendency to disappear even after 9 months. Aside from pulmonary toxicity, schedules C and D reduced the degree rather than the frequency of side effects. With schedule E, on the contrary, stomatitis occurred only in 25% of cases, alopecia in 40% and sclerotic changes in the skin of hands in 70%. Excluding fever, nausea and vomiting, for all dose schedules the first sign of toxicity occurred in the large majority of patients within the 4th and the 6th dose. The minimal toxic dose appears to be 60–75 mg/m^2 (Table 2).

Pulmonary toxicity was defined on clinical bases by the occurence of rales, ronchi and, occasionally, of pleural friction rubs in patients without clear-cut signs of pulmonary infection or heart failure or progression of neoplastic lesions in the lungs. If pulmonary toxic lesions

*0–0: no response; 0–A: only subjective benefit; 0–B: objective changes without subjective benefit; 0–C: subjective and objective benefit for less than 1 month; 1–A: subjective and objective benefit for more than 1 month; 1–B: tumor regression greater than 50% for more than 1 month in an asymptomatic patient.

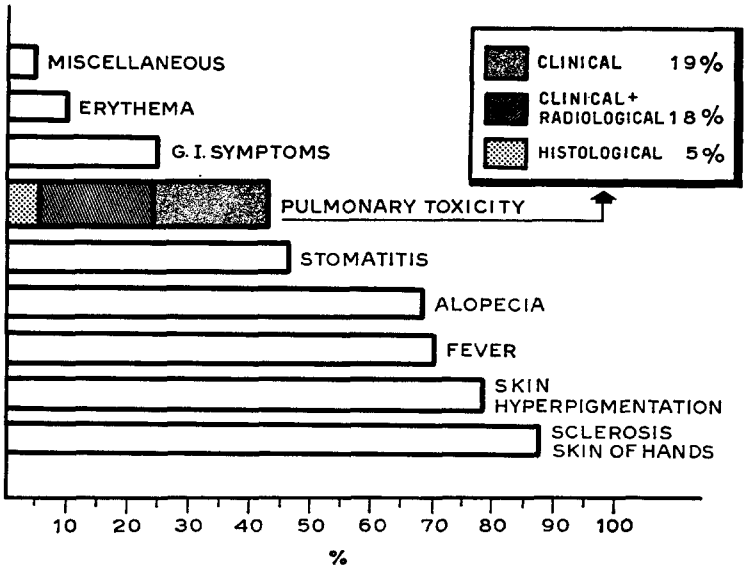

Fig. 1. Manifestations of toxicity in 154 patients after a complete course of i.v. bleomycin (dose schedules A, B, C, D and E).

progress, the patients may complain of dyspnea which occasionally ends in a progressive respiratory insufficiency not responsive to supportive drug therapy.

Figures 4, 5 and 6 show the pattern of pulmonary toxicity as can be detected on chest X-ray. Early toxicity appears as a fine reticulonodular pattern mostly located at the lower lung zones and at the costophrenic angle. In more advanced stages, the nodularity becomes widespread and mixed with patchy infiltrates. The pulmonary lesions are unassociated with mediastinal or hilar lymph node enlargement. Pleural effusion is very uncommon. Upon prompt discontinuation of the treatment and institution of therapy with corticosteroids and antibiotics, a gradual regression to a complete roentgenographic resolution can be observed within 1–4 months.

Figures 7, 8 and 9 show the histopathological appearance of lung lesions attributable to BLM toxicity. Initially, there is edema of the alveoli and interstitial tissues. Swelling of alveolar lining cells with or without desquamation is present as well as moderate infiltration of macrophages. Large mononuclear or binuclear cells can also be observed. The alveolar space appears filled by an amorphous proteinaceous material resembling a hyaline membrane.

Subsequently, alveolar collapse occurs and interstitial fibrosis becomes evident. The fibrosis can be focal or diffuse. A superimposed bronchopneumonia occurs frequently. Outside of the lungs, no other lesions compatible with bleomycin toxicity were seen with the exception of the skin, where post-BLM skin biopsies showed increases in collagen deposition.

During the first part of this trial, pulmonary toxicity was somewhat overlooked both clinically and radiologically and the diagnosis was made only retrospectively. Therefore, 10 patients received a further dose of BLM while pulmonary damage was already present. Table 3 summarizes the course of pulmonary toxicity as observed in 65/154 patients (42%) treated with adequate doses of BLM. In 30/65 cases (46%) pulmonary toxicity consisted almost always in clinical findings. Chest X-ray was only suggestive of "bronchitis" but the reticulonodular pattern typical of the interstitial lesions was absent. While 7 patients were lost to follow-up, the physical findings regressed in 7 cases, they remained unchanged in 10 and progressed in 5. In 28/65 cases (43%) the clinical findings suggestive of pulmonary toxicity were confirmed radiologically a few days later on repeated roentgenograms. More precisely, in 4/28 patients the signs of pulmonary toxicity were present only on chest roentgenograms without clear-cut association of rales. Again, during the follow-up period the lesions either regressed (9 cases) or remained unchanged (9 cases) while in 10 patients both clinical and radiological progression were observed. In 7/65 patients (11%) post-mortem examination detected various grades of pulmonary toxicity, focal in 4 and diffuse in 3. In only 3/7 cases clinical and radiological signs of lung toxicity were present. In all 3 patients the histologic sections of the lungs revealed diffuse collagen deposition.

As far as the presumptive cause of death is

Table 3. Type and course of pulmonary toxicity in 65 cases

Diagnosis	No. cases	Follow-up	Cases	Presumptive cause of death
Clinical	30 (46%)	lost to follow-up	7	
		regression	7	advanced cancer 3
		no change	11	cancer + BLM? 3
		progression	5	BLM? 3
Radiological or Clinical + Radiological	28 (43%)	regression	9	
		no change	9	advanced cancer 2
		progression	10	cancer + BLM 5
				BLM? 3
Histopathological*	7 (11%)			advanced cancer 4
				cancer + BLM 1
				silicosis + BLM 1
				BLM 1

*In 4 other cases post-mortem examination failed to detect pulmonary toxicity.

concerned, no definite conclusions can be drawn in some patients since they died outside the hospital. However, in 9 cases the cause of death can be related to progression of cancer in the lungs or in other organs, while in 9 BLM might have played a role in determining the fatal issue together with progressive cancer. In 8 patients, including a 68-year old man with a longstanding history of silicosis, a clear-cut cause of death could not be found. Since pulmonary symptoms and signs were severe and progressive, we assume that BLM might have been responsible for the irreversible respiratory insufficiency. In 2 of these patients the microscopic examination of the lung tissue showed diffuse interstitial fibrosis. Therefore, the mortality rate related to BLM toxicity accounts for about 5%.

Table 4 reports pulmonary toxicity in relation to the 5-dose schedules and the total dose-producing lung toxicity. With the first 3 schedules the incidence of pulmonary damage varied from about two-thirds to about half of cases. With schedules D and E the incidence was reduced to about 30% of patients. This side effect occurred also after doses less than 100 mg/m² (minimum 75 mg/m² as detected at post mortem examination). Symptoms appeared usually after 4 to 10 weeks from starting therapy.

Table 4. Pulmonary toxicity in relation to dose schedules and total doses

Dose schedule	Toxicity		Mean total toxic dose (mg/m³)	Onset (days)	
	Cases	%		From starting BLM	From last dose
A	12	66	359	71	10
B	10	55	283	55	8
C	15	48	135	70	17
D	23	33	132	50	9
E	17	29	93	28	13

In Figs. 10 and 11 pulmonary toxicity is analyzed in relation to previous lung disease as well as in relation to the age of the patients and to the total dose of BLM administered. It appears evident that in our case material the incidence of drug-induced pulmonary lesions was not significantly different in the group of patients with a completely negative chest roentgenogram and those with either chronic pulmonary diseases (e.g. emphysema, chronic bronchitis, localized fibrosis post t.b.) or with neoplastic lesions in the lung. Furthermore, no clear-cut relation could be found between the age of the patients and the total dose

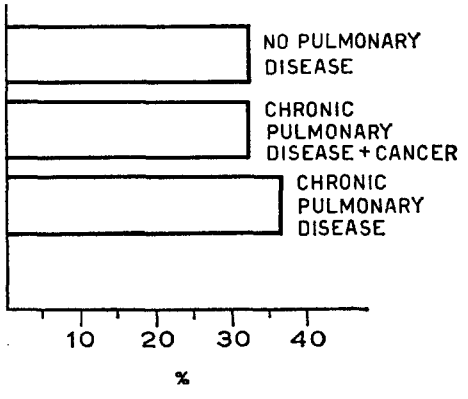

Fig. 10. Pulmonary toxicity in relation to previous lung disease (65 cases).

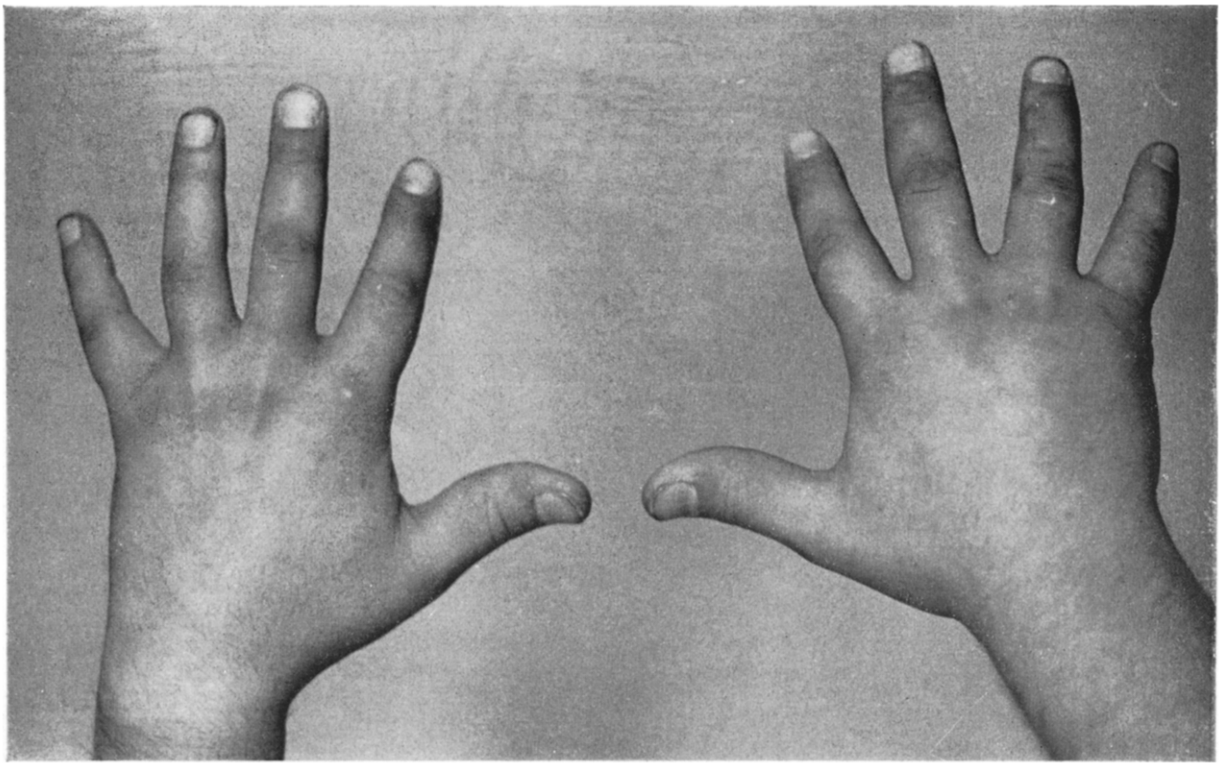

Fig. 2. Typical sclerotic changes of the skin of hands with edema of the fingers.

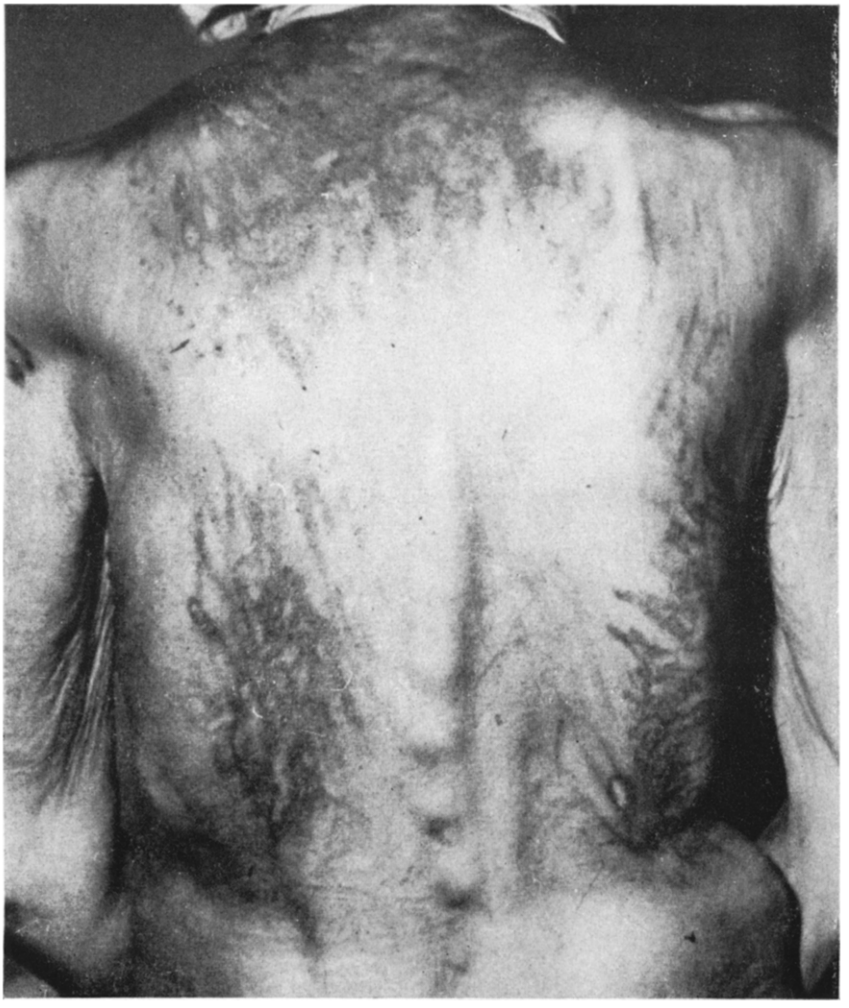

Fig. 3. Typical hyperpigmented stria of skin.

(to face p. 210)

Fig. 4. *Posteroanterior roentgenogram. The magnified view reveals a fine reticulonodular pattern in the lower portion of the right lung. Early pulmonary toxicity bleomycin induced* (*total* 90 mg/m^2).

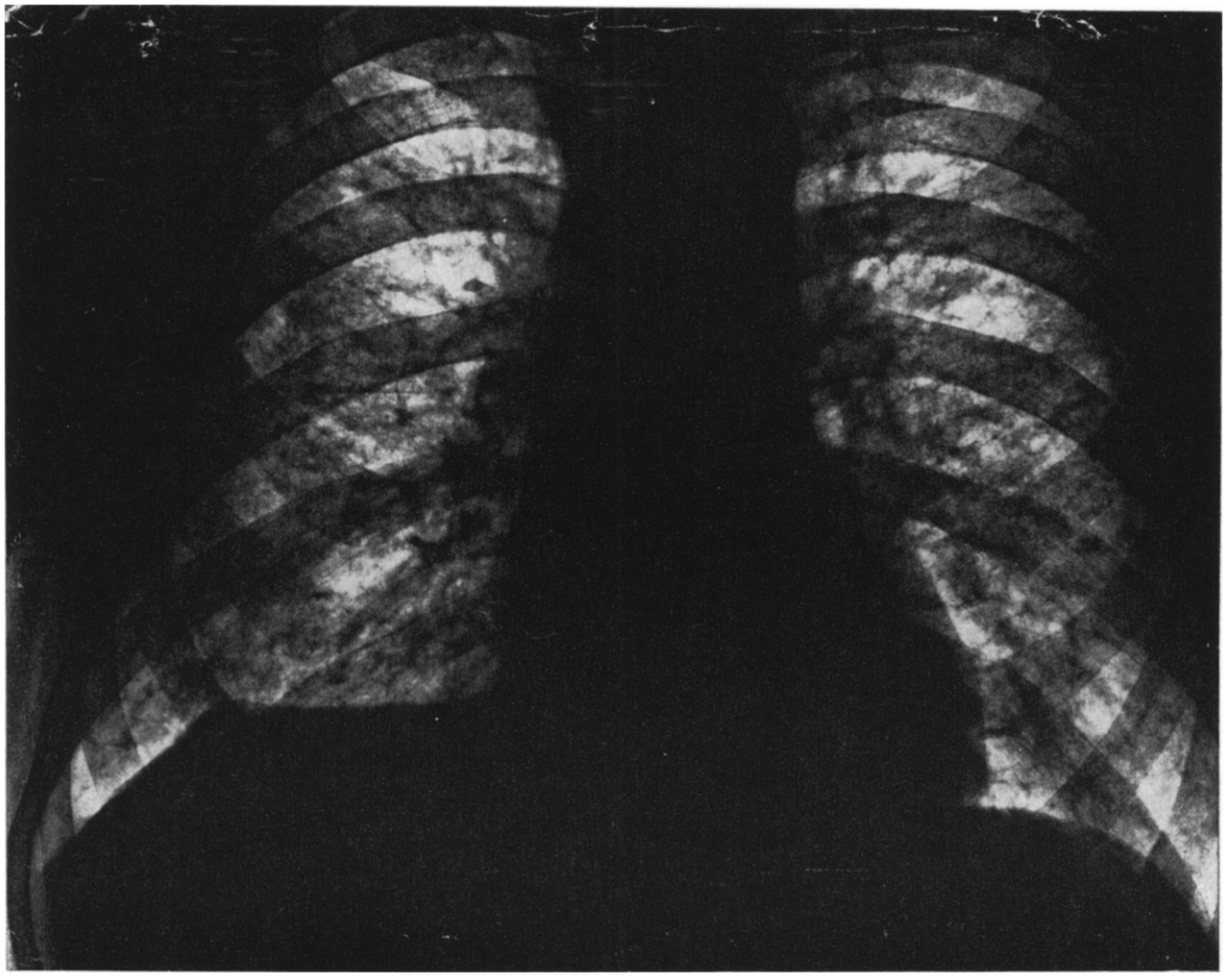

Fig. 5. *Posteroanterior roentgenogram showing a bilateral diffuse coarse streaking nodularity after* 150 *mg/m²* *of bleomycin.*

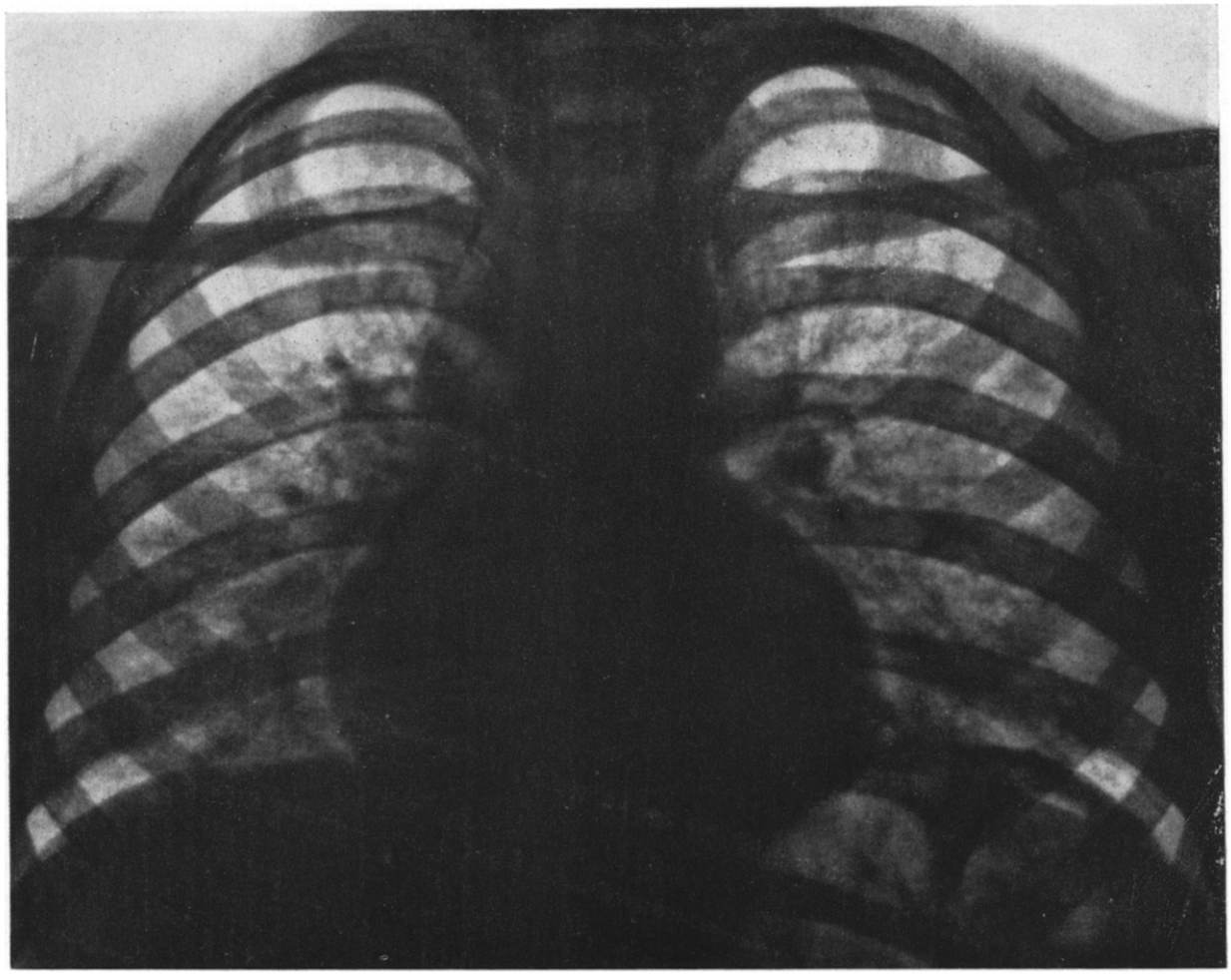

Fig. 6. *Posteroanterior roentgenogram. A coarse reticular pattern with patchy infiltrates is present throughout both lungs after* 225 *mg/m²* *of bleomycin. Histologic sections obtained at post mortem examination revealed diffuse alveolar collapse and interstitial fibrosis* (*see also Fig.* 9).

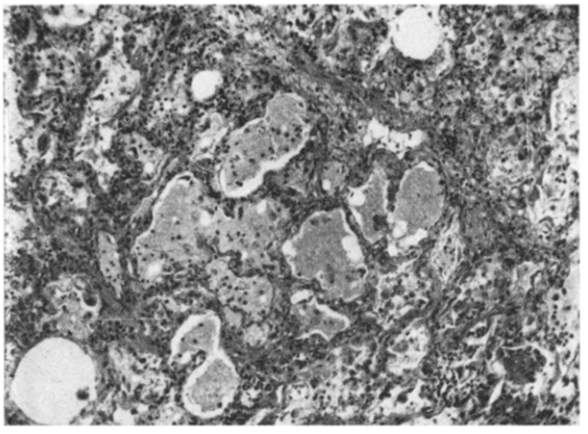

Fig. 7. Photomicrograph of the lung of a 25-year old man with Hodgkin's disease after 75 mg/m² of bleomycin. Edema of the alveolar walls and presence of large mononuclear cells. The alveolar spaces are filled by a proteinaceous material. Hematoxilin and eosin (× 120).

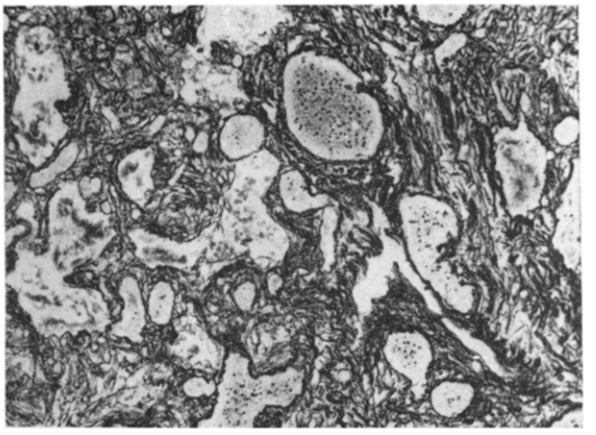

Fig. 8. Photomicrograph of the lung of the same patient showing thickening of the alveolar wall sand septa due to marked increasing of reticulin fibres. Silver stain (× 120).

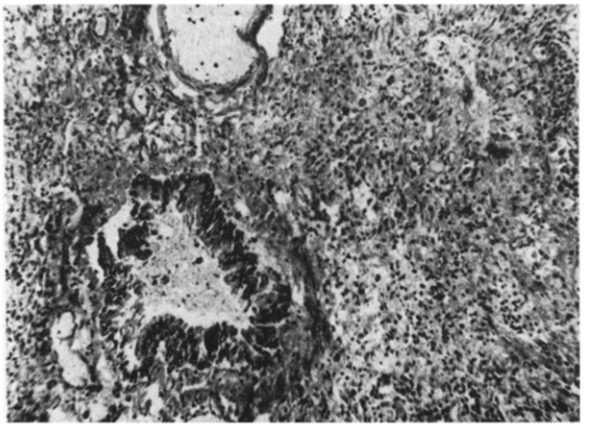

Fig. 9. Photomicrograph of the lung of a 7-year old boy with acute lymphosarcoma cell leukemia after 225 mg/m² of bleomycin. The normal pulmonary pattern is replaced by a proliferation of fibroblasts and histiocytes. Hematoxilin and eosin (× 120).

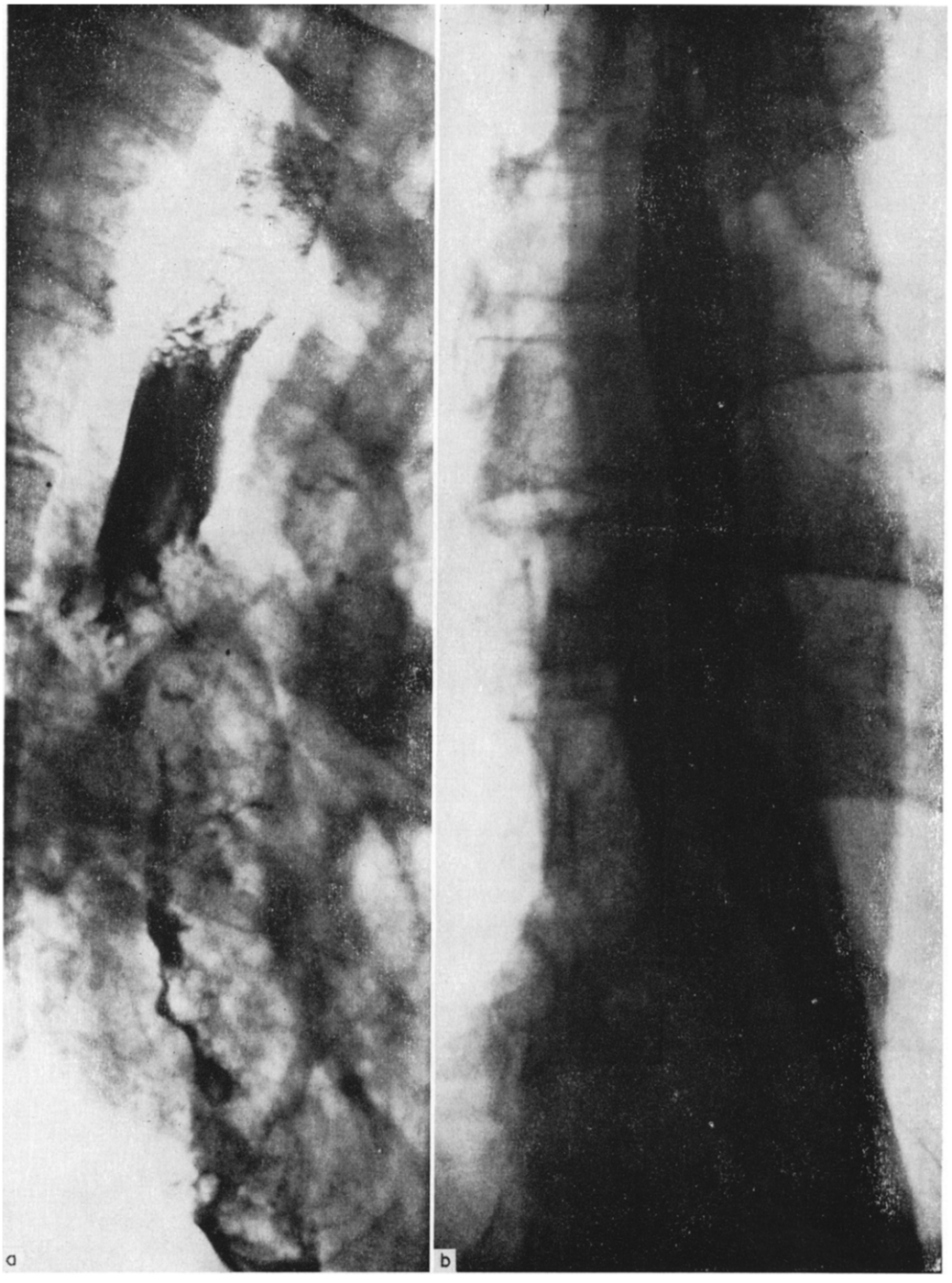

Fig. 12. (a) *Lateral view. Extensive carcinoma of the esophagus.* (b) *Slightly oblique view. Marked regression after* 180 *mg/m²* *of bleomycin.*

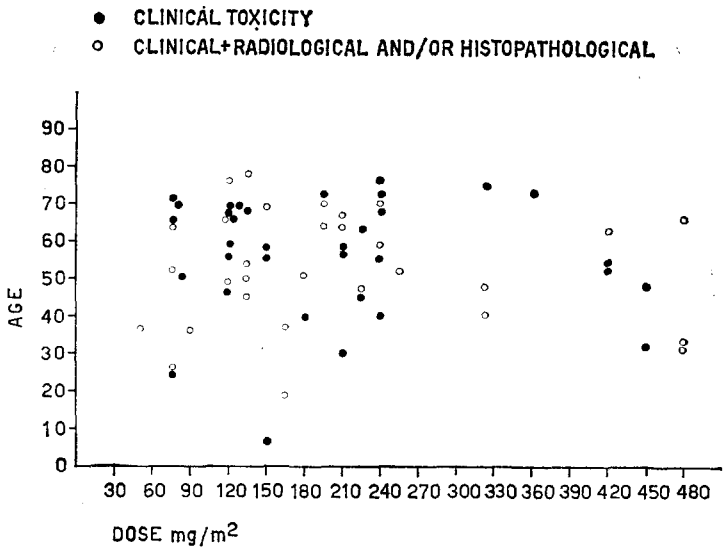

Fig. 11. *Pulmonary toxicity in relation to age and total dose of bleomycin* (65 *cases*).

causing pulmonary toxicity, although the incidence of pulmonary damage appears slightly higher in patients over 50 years.

From our experience BLM appeared devoid of significant changes in the bone marrow elements, in the liver function tests as well as in the blood urea nitrogen, serum uric acid and serum electrolites.

Therapeutic responses

Table 5 summarizes the results obtained in various types of lymphoprolipherative disorders and in epidermoid carcinomas. In malignant lymphomas, 40% of the patients showed a category I response and in 3 cases a complete remission was achieved. Furthermore, 2/4 cases with acute lymphosarcoma cell leukemia responded promptly to a relatively low dose of BLM with a good partial and, respectively, complete marrow remission. The 7-year old boy in whom a complete remission was obtained, died subsequently because of progressive lung toxicity. Post mortem examination failed to detect lymphosarcoma cells not only in the marrow but also in lymph nodes, liver and spleen which were markedly enlarged before therapy. Satisfactory responses were seen in head and neck tumors (56% of category I) and in carcinoma of the esophagus (60% of category I) (Fig. 12). On the contrary, no significant responses were observed in chronic lymphocytic leukemia and in epidermoid carcinoma of the lung.

Table 5. *Therapeutic response to bleomycin in lymphomas and epidermoid carcinomas*

Neoplastic disease	Appraisable cases	Categories of responses (Karnofsky)						
		0–0	0–A	0–B	0–C	1–A	1–B	Complete
Hodgkin's disease	17	5	2	1	1	5	1	2
Lymphoreticular sarcoma	12	3	1	1	3	1	2	1
Ac. lymphosa. cell leukemia	4	2				1		1
Chronic lymphatic leukemia	3	2		1				
Head and neck cancer	48	7	4	4	6	18	7*	2
Esophagus cancer	10	1		1	2	4†	1	1
Lung cancer (epidermoid)	10	8		1		1		
Skin cancer	5				1	2	2	
Cervix cancer	2		1			1		
Glans cancer	2				1	1		
Total	113	28	8	9	14	34	13	7

*1 patient had also a cancer of the esophagus (1–A).
†1 patient had also a cancer of the larynx (1–A).

Table 6. Therapeutic response to bleomycin in miscellaneous tumors

Neoplastic disease	Appraisable cases	Categories of response (Karnofsky)					
		0–0	0–A	0–B	0–C	1–A	1–B
Lung cancer	5	2	1	2			
Breast cancer	6	4	1		1		
Nasopharynx cancer	2					1	1
Testicle cancer (embryonal)	2	1				1	
Thyroid cancer	3	2				1	
Kidney cancer	1	1					
Parotid cancer	1						1
Choriocarcinoma uterus	1					1	
Unknown primary cancer	1		1				
Melanoma	4	1		2	1		
Mesothelioma	2		2				
Kaposi's sarcoma	1					1	
Soft tissue sarcoma	4	2	1	1			
Total	33	13	6	5	2	5	2

Table 6 reports the therapeutic results obtained in 33 patients with miscellaneous types of tumors. Only scattered responses were seen, although the number of cases for each tumor category is too small to draw conclusions. It should be pointed out, however, that 2/2 patients with nasopharyngeal carcinoma showed a category I response. The patient with psoriasis had no appreciable response to BLM. On the contrary, this disease showed a moderate regression for a period less than 1 month duration in 2 cases who had Hodgkin's disease and respectively lymphoreticular sarcoma.

The over-all regression rate for categories 0–C, 1–A and 1–B is 53 %, for category I 41 %. Complete remissions occured in about 5 % of the cases. In responsive patients, regressions were usually prompt (1–3 weeks) and the mean therapeutic dose was 60 to 70 mg/m^2. The duration of regression, however, was brief with or without maintenance treatment. In 32 patients it lasted less than 1 month (categories 0–B and 0–C) and in 62 patients with a category I response the average duration of regression was 2 to 3 months (in 1 case 12 months). No particular difference in the incidence of response to BLM was seen between the group of pretreated and that of untreated patients.

Table 7 shows the therapeutic response in relation to the 5 schedules employed. This was evaluated only in the largest group of patients treated with BLM (head and neck carcinomas). The incidence of category I regression varies in the different schedules. However, it appears that satisfactory responses can be obtained also with a low intermittent dosage (schedule E).

Table 7. Response in relation to the dose-schedules Head and neck cancer: 48 cases

Dose schedule	Response (category I or complete)	
	No. responsive cases	%
A	3/7	43
B	4/6	66
C	4/6	66
D	13/26	50
E	2/3	66
Total	27/48	56

DISCUSSION

In recent years considerable progress has been achieved in the control of neoplastic disease by new antitumor antibiotics. Streptonigrin methyl ester, mithramycin, daunorubicin and adriamycin have been shown in fact to be active against a number of tumors resistant to conventional agents. Moreover, some of these drugs proved to be specifically effective in some forms of neoplasia [27–29]. The therapeutic response to all these new agents is usually accompanied by unusual, severe and occasionally life-threatening side effects. Subsequent investigations however have led to the development of dose-schedules which substantially reduced toxicity while maintaining unaltered the therapeutic effect [28, 29].

Bleomycin is a new growth-inhibiting antibiotic which is presently under clinical investigation in many centers. Its therapeutic effect seems to be specific against different types of

epidermoid carcinomas. However, the development of pulmonary toxicity after therapeutic doses appears to be higher than previously reported by most Japanese and European investigators. Therefore, further studies to develop adequate therapeutic programs are requested.

Our phase I and preliminary phase II evaluation of BLM lead to some considerations which can be summarized as follows:

(1) The optimal dose-schedule has not yet been established because of early toxicity with all dosages used and, especially, because of delayed pulmonary toxicity.

(2) High bi-weekly doses and daily administration of BLM can produce cumulative toxicity leading to early pulmonary damage.

(3) When carefully and systematically searched, the signs of pulmonary damage appear to constitute a considerable percentage of all toxic manifestations.

(4) Besides in some epidermoid carcinomas, BLM appears to be particularly effective in all types of malignant lymphomas.

The difficulty in determining an adequate and safe therapeutic regimen of BLM has been mostly represented by the fact that with all dose schedules employed in our trial, side effects and particularly pulmonary toxicity appeared after a relatively short period of time. Schedules D and E however, decreased substantially the incidence of pulmonary complications, suggesting that small bi-weekly doses (10–15 mg/m^2) are to be preferred to equivalent daily dosages. This intermittent regimen would be more in accordance with the results of disposition studies showing that most of the injected drug is excreted within 24 hr [8, 9]. However, even with this moderate therapeutic regimen, pulmonary toxicity occurred in about 30 % of our cases and in 50 to 80 % of patients reported by recent Japanese investigators [16–18]. Different degree and extent of drug-induced pulmonary lesions were described recently in 6/18 (33 %) of cases by Cunningham *et al.* [25] who administered 20 mg/m^2 twice weekly or 10 mg/m^2 weekly, doubled every two weeks. A higher incidence of pulmonary toxicity was observed by Luce *et al.* [23] who injected BLM at 25 to 35 mg/m^2 for 5 to 28 days.

All these data confirm first of all that pulmonary damage induced by BLM can occur even with small doses. Some of our cases became toxic after 60 to 75 mg/m^2 (in our patients this was confirmed at post mortem examination) and in Yagoda's series [22] one patient died after a total of 49 mg (autopsy report). Furthermore, although high and especially i.v. daily doses undoubtedly increase the incidence of lung complications, BLM pulmonary toxicity seems to be somewhat schedule-independent and, at least from our experience, not strictly related to the age of the patients or to the presence of chronic lung diseases. Finally, symptoms and signs usually appear days or weeks from the last dose (delayed toxicity).

The diagnosis of lung toxicity, although at times difficult to establish, can be made through experience on physical examination, chest X-rays and autopsy. The detection of fine rales at the base of one or both lungs should always raise the suspicion of initial pulmonary damage. If the drug is stopped promptly, the lesions can remain at their early stage, so they do not even become visible on repeated roentgenograms. Serial chest X-rays show the presence of early interstitial lesions in about 50 % of cases 1 to 2 weeks after the discovery of crepitations. When detected in time, both clinical and radiological signs may either remain unchanged or gradually regress to a complete disappearance within 1 to 4 months. On the contrary, if drug administration is pursued, pulmonary lesions become symptomatic and inexorably progressive. As also reported by other authors [17, 22, 23, 25], great variability in the degree and extent of lung lesions has been observed at microscopic examination. Minimal or focal lesions in different stages of evolution are found throughout the lungs but mostly at the bases, even in absence of previous physical or radiological evidence. In these cases their presence can be suspected through a pulmonary function test by a decrease in some of the parameters [22, 23].

In summary, the incidence of pulmonary toxicity seems to be higher than previously reported, but its course appears benign provided the drug administration is promptly arrested, although in very a few cases small doses can produce fatal respiratory insufficiency. Physicians should be aware of the fact that early lung toxicity can be detected through the stethoscope, and once pulmonary signs have occurred and subsequently disappeared, a further course with BLM is, at the moment, not recommended even in the presence of considerable tumor response. Pulmonary toxicity with interstitial lesions occurs also after Methotrexate and busulfan, but its actual incidence has not yet been established [30–32].

From the therapeutic point of view, our clinical trial confirms the efficacy of BLM in carcinomas of head and neck, and of the esophagus. Furthermore, the drug appears definitely effective in all types of malignant

lymphomas also when malignant cells are present in the bone marrow and in the peripheral blood. On the contrary, our experience does not prove, with all doses schedules tried, that epidermoid carcinomas of the lung are responsive to BLM. In a number of other tumors therapeutic results have been observed but, so far, no definite conclusions can be drawn. Although small doses are usually required to produce a therapeutic response, the duration of regression appears to be short-lived. Therefore, the drug does not seem to be useful for maintenance treatment. No cross resistance with radiation therapy, or with most of the conventional anticancer drugs has been observed. Our results are in accordance with those recently reported by American investigators. [22, 23, 25].

In conclusion, BLM is a new antitumor antibiotic characterized by a peculiar toxicity, by absence of significant hematologic, renal or hepatic toxicity, and by a specific activity on some epidermoid carcinomas. Both optimal dose-schedule and pulmonary toxicity should be better investigated in future trials. However, severe pulmonary complications can be minimized by low intermittent dosages and early diagnosis, probably by corticosteroids and possibly by intramuscular route. Because of its toxic and therapeutic properties, BLM appears suitable for therapeutic trials in short courses alone or in combination with myelo-suppressive agents.

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Electrophoretic Pattern of Sera of Normal and Cancer Patients

J. MAISIN, P. COUVREUR, I. P. OCHRYMOWICZ and E. VAN DUYSE
Institut du Cancer, Louvain, Belgium

Abstract—*Acrylamide disc electrophoresis patterns of serum from cancer patients are compared with those of normal serum.*

A significant difference in pattern is observed, especially when sera are incubated, prior to electrophoresis, with total yeast RNA. The results obtained with this technique are then compared with a complement fixation reaction using as antigen the same yeast RNA. It was found that the electrophoresis technique has a higher percentage concordance with histological diagnosis.

However, certain anomalous results were obtained and these are discussed.

MAISIN and co-workers have already shown that total ribonucleic acid extracted from the β strain of Saccharomyces Cerevisiae seems to possess antigenic properties which may be used in a complement fixation reaction to identify serum from cancer patients [1–3].

In this reaction, the serum under investigation is diluted 1/10 in veronal buffer (pH 7·2) and incubated for 30 min at 37°C with 250 γ of the RNA, diluted in the same buffer. Then the usual hemolytic system (i.e. sheep erythrocytes and hemolysin) is added and the mixture reincubated for 30 min at 37°C.

In the case of serum from clinically confirmed cancer patients, hemolysis takes place, whereas with normal sera the red blood cells precipitate. This suggests that the complement has been absorbed or fixed on the complex of the RNA with some factor (antibody ?), present in normal serum; the complement in the serum from cancer patients remaining apparently unifixed for reasons which are not yet fully understood.

The RNA antigen used in this reaction is extracted from a 48 hr culture of Saccharomyces Cerevisiae by Crestfields phenol procedure [4] after prior disruption in a French Press. This extract, apart from RNA, contains inevitably some glycogen.

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Pure glycogen, obtained by gel filtration on sephadex G200, after prior hydrolysis of the RNA by ribonuclease and 20% sodium hydroxyde, was tried but was found to be devoid of any specific activity. This suggests that the active component is the RNA.

In order to try to understand the mechanism of this reaction and to find out the reasons for the differences of behaviour of normal and cancer sera towards this RNA β antigen, we proceeded to analyse the two types of sera, using the polyacrylamide gel disc electrophoresis technique of Ornstein and Davis [5].

The electrophoresis takes place in tubes containing a small pore 7·5% separating gel, followed by a large pore stacking gel and over this the sample is mixed with large pore gel and polymerised.

A current of 4 mA per tube is passed and a tris-glycine electrolyte buffer is used. The electrophoresis takes 90 min. The gel is removed and fixed and stained in a 1% solution of Amido black dissolved in 7% acetic acid. This is followed by electrophoretic destaining. It must be stressed that to have valid and steady results, the serum must be fresh or kept refrigerated, and certainly not kept more than 6 hr at room temp. The electrophoresis patterns of most normal individuals show systematically 4 distinct bands above the transferrin band

which are easily identified (Figs. 1 and 2). However in a few cases both normal and pathological sera showed marked differences in the general pattern, (Fig. 1) and these were not considered. In cancer patients, band 3 is generally absent or in a small number of cases is replaced by 2 faint bands (Figs. 1 and 2).

We have examined the sera of 59 normal individuals using both techniques, namely the method of complement fixation on the RNA antigen and serum factor, and the electrophoresis method.

Among the 59 sera, 48 or 81% gave a normal reaction (i.e. absence of hemolysis), and 11 gave an abnormal reaction (i.e. hemolysis occurred).

With the electrophoretic method, 46 (78%) sera gave normal patterns (i.e. presence of band 3), the rest gave an abnormal pattern. Thus with normal sera both methods give similar results.

When we consider the behaviour of sera of cancer patients, towards the complement fixation reaction described above and the disc electrophoresis method, it is important to classify those patients according to their staging; early cases (stage 0 or stage IT_1N_0), moderately advanced cases (stage II and stage III) and late cases (stage IV).

We have already published [2] the results obtained with the complement fixation reaction on 333 cases which were moderately advanced cases (stage II and III) giving 73% of correct results, that is to say, a hemolytic type of reaction. None of those patients however were examined with the acrylamide disc electrophoresis method.

If we now compare the results obtained with both methods, (i.e. the complement fixation reaction and the disc electrophoresis method), on two groups of cancer patients; a first group of 83 cases belonging to stage 0 and stage I, and a second group of 35 advanced cases (stage IV), then out of those 83 cancer sera, 43 or 51% give a reaction of the hemolytic type, with the complement fixation reaction, while with the electrophoretic technique, 75 patients or 91% give a pattern characterised with the absence of band 3. Practically, it means that with the electrophoretic technique in 91% of the cases, we should have been able to detect the abnormality corresponding to cancer sera while the reaction of complement fixation gives only 51% of correct results.

With the sera of 35 cases, belonging to the second group (advanced cases), 48,6% of those sera give a reaction of the hemolytic type by the complement fixation reaction, while 86% give an absence of band 3 with the electrophoretic technique. There is then a marked difference between the two techniques, the electrophoretic technique giving by far the better results, which correspond to the histological diagnosis of cancer.

For some unknown reason, certain advanced cases of cancer, or some very early cases, give a sedimentation of red blood cells with the complement fixation reaction, in other words, a reaction corresponding to the reaction of healthy individuals, thus a false result.

It appears clearly that the electrophoretic technique is able in a large percentage of cases to make a better distinction between normal and cancer sera, even if those sera belong to patients suffering from advanced cancer or to patients with very early cancer.

We then studied the effect of the addition of the RNA antigen on the electrophoretic pattern of normal and cancer sera.

Thus prior to electrophoresis, we add the RNA antigen to the serum to be examined and we incubate the mixture at 37°C for 30 min.

The results of this action of the antigen are clearly shown in Fig. 3, giving the patterns obtained with different types of sera with and without antigen, and in Fig. 4 showing schematically these same patterns.

It is obvious that, on addition of the antigen, band 3 of normal sera disappears whereas there is no apparent change in the pattern of sera of cancer patients, even for a few cases of cancer showing a faint band 3.

Finally, if we compare the diagnosis value of the complement fixation reaction with the electrophoretic method both on normal sera and all the cancer sera of this series, including advanced and very early cases, we find the following figures:

Sera normal individuals	59 cases	Pooled sera of cancer patients 118 cases
Complement fixation reaction test	81%	43%
Electrophoresis	78%	89%

These figures show that for the identification of normal sera both tests are giving similar results, reliable in 78 to 80% of the cases. However, for the identification of sera from early and advanced cases, we notice an important discrepancy between the two methods in

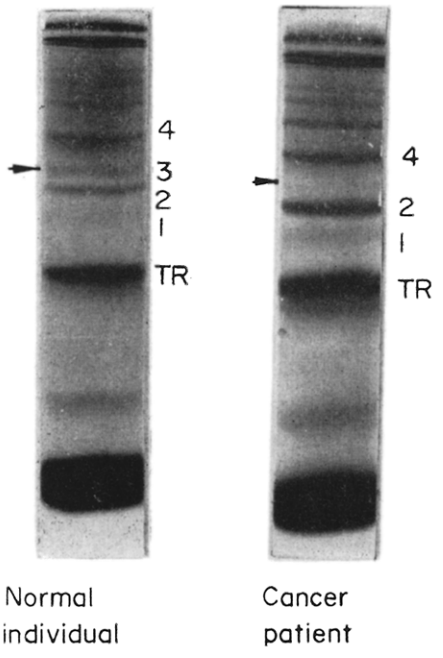

Fig. 1. Comparison between the electrophoretic pattern of a normal serum and the serum of a cancer patient. The serum of a normal individual shows distinctly several bands above the transferrin (TR.) band. The first 4 and mainly band 3 are of particular interest. In the serum of the cancer patient, band 3 is absent or very strongly attenuated.

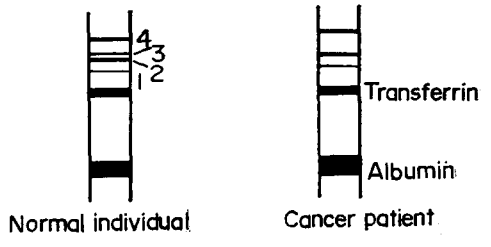

Fig. 2. This figure represents diagrammatically the presence of band 3 above the transferrin in the serum of a normal individual and its absence in the serum of a cancer patient.

(to face p. 218)

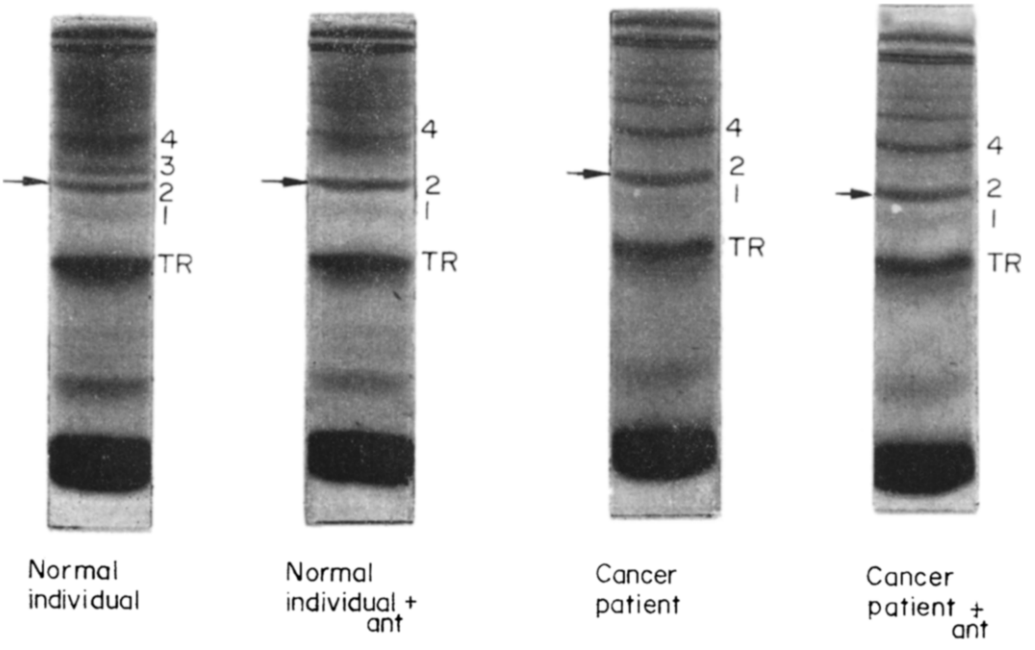

Fig. 3. Comparison of the influence of the RNA antigen on the electrophoretic pattern of the serum of a normal individual and on the serum of a cancer patient: under the influence of the RNA antigen, band 3 disappears in normal serum. Whereas the addition of RNA has no influence on the pattern of the serum of a cancer patient.

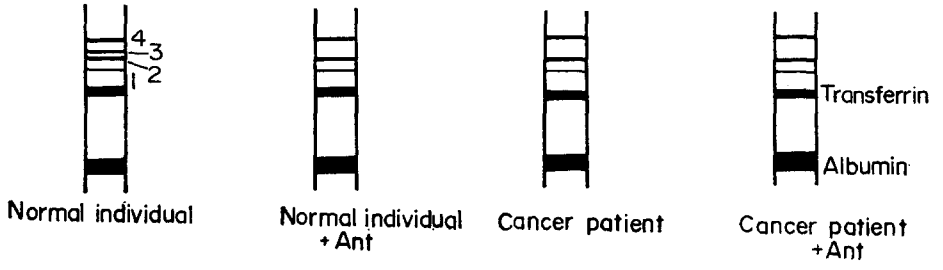

Fig. 4. This figure gives schematically the phenomenon represented in Fig. 3: the influence of RNA antigen on the electrophoretic pattern of a normal serum and the serum of a cancer patient.

favour of the electrophoretic method: 43% against 89%.

It is interesting to add that we have also examined the sera of five cases of lymphosarcoma, one case of chronic lymphocytic leukemia, and one case of plasmocytoma. All the electrophoretic patterns indicated a pattern typical of a cancer patient, whereas only two adequate diagnoses were made with the serological reaction.

The exact nature of band 3 is as yet unknown. It may be either the breakdown product of complement C'3 (B1C/B1A) [6, 7] or an α_2-lipoprotein [8]. The problem is under current investigation.

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Recent Progress in Bladder Cancer

D. B. CLAYSON and E. H. COOPER
The University of Leeds, Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds LS2 9NL, England

An international conference on bladder cancer was held in Leeds, under the auspices of the International Union Against Cancer, from 22 to 24 September, 1971. Some fifty clinicians and scientists from fourteen countries reported and discussed recent work on aspects of bladder cancer in man and animals.

CLINICAL RESEARCH

THE EXTENT, variety and natural history of neoplastic disease involving the urothelium in man is extremely diverse. It ranges from the simple solitary benign "papilloma" to multicentric invasive tumours appearing in several sites in the urinary tract. The problem that faces pathologists and surgeons is how to recognize those patients, presenting initially with a tumour of relatively low malignancy, who will in due course develop a more aggressive form of the disease. Unfortunately, there still seems to be a considerable divergence of opinion as to what criteria indicate that a well differentiated papillary tumour may be the forerunner of a far more malignant tumour. Melamed described how the changes of carcinoma *in situ* occurring focally in papillomas may be a step in their evolution towards an invasive tumour. An appeal (Mostofi) for a more uniform system of classification ran into considerable difficulty due to national and personal differences on the precise definition of the terms commonly used to describe tumours.

Electron microscopic studies focussed attention on the properties of the luminal membrane of urothelium which in normal mammals is asymmetric and in the rat has been shown to be composed of a lattice of sub-units. Hicks *et al.* reported studies on the chemical and physical composition of this membrane and how its structure could be altered by relatively mild chemical attack. The surface topography of human bladder epithelial cells and bladder tumours has been revealed using scanning electron miscroscopy (Fulker). The normal cell surfaces are ridged; well differentiated tumours have microvilli, and poorly differentiated tumours are almost smooth. The cell adhesion of well differentiated tumours resembles that of normal bladder epithelium with tight junctions at the luminal margin and marked cellular interdigitation; with dedifferentiation, the contact between cells becomes far less intimate. This observation appeared to link with the report that the *in vivo* permeability of human bladder epithelium to sodium and water is unchanged by the presence of a well differentiated tumour, but is increased when a poorly differentiated tumour is growing in the bladder; infection causes a marked rise of permeability, a fact that should be remembered when considering topical chemotherapy (Fellows).

The technical problems of autoradiographic studies of ^{3}H-thymidine incorporation *in vitro*, as a guide to tumour malignancy, were thought to be a limitation to its usefulness, although the incorporation of RNA precursors looked to be more promising (Veneema *et al.*). Stathmokinetic tests using colcemid to block mitosis *in vivo* provided good evidence of the high rates of cell loss in all forms of bladder tumour, being at its peak in the anaplastic types; the bulk of the cell loss occurred within the tumour and not by exfoliation from its surface (Spooner and Cooper). These workers showed that well and moderately well differentiated tumours had modal chromosome numbers in the 44–49 range, whilst poorly differentiated tumours usually had much higher chromosome numbers,

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although 20% were found to be still within this diploid range. Some well differentiated tumours exhibited no detectable chromosome abnormality. Recurrent tumours showed that some abnormal clones were fairly stable and could be recognized on several occasions by their characteristic marker chromosomes. However, chromosome number and karyotype were not readily correlated with the state of differentiation.

The importance of carcinoma *in situ* as part of the general syndrome of urothelial instability was stressed by both surgeons and pathologists. The practice of biopsying apparently healthy epithelium at random in cancer-bearing bladders, has revealed an unexpectedly high incidence of carcinoma *in situ*. The follow-up studies of workers exposed to 4-aminobiphenyl (Koss) indicated how intervals of more than 30 months can occur between a positive cytological diagnosis of urothelial cancer and its clinical detection; carcinoma *in situ* is considered to be the source of the malignant cells in the urine during this interval. Spontaneous regression of carcinoma *in situ* has been observed. It is still an open question whether this condition is always the precursor of bladder cancer, or whether some tumours arise as the result of change in hyperplastic papillary lesions.

Bubenik *et al.* and O'Toole *et al.* described their experiments on the study of the antigenicity of human bladder cancer cells *in vitro* and the cytotoxic effect of lymphocytes from bladder-cancer patients on autochtonus and allogeneic bladder cancer cells. The sera of some bladder-cancer patients contain cytotoxic and blocking antibodies. Lymphocyte cytotoxity disappeared rapidly with the start of radiotherapy of a bladder cancer but returned soon after the course was completed, and has remained active for 3 to 4 months. Patients who had been treated with radiotherapy $1\frac{1}{2}$ to 10 years previously showed no lymphocyte toxicity except in cases of tumour recurrence. This change of emphasis from analysis of the behaviour of tumour structure to investigation of host-tumour relationships may prove to be an important milestone in bladder cancer research. Bladder cancer is a disease whose natural history is now well known, but explanations are still required for many of the phenomena we observe in man and laboratory animals.

EPIDEMIOLOGY

Less than 1% of all bladder tumours, even in highly industrialized countries, can be attributed directly to exposure to the recognized carcinogenic aromatic amines in the chemical, rubber and electric cable-making industries. Evidence was presented that people working in a few other occupations in Leeds, England (Anthony and Thomas), Boston, U.S.A. (Cole *et al.*), and Stoke-on-Trent, England (Veys) develop 20 to 30% of the total bladder cancer incidence. The Leeds survey clearly showed that a period of 20 years or more in the newly suspect occupations was required for the induction of bladder cancer, but there was no indication whether this exposure was to low levels of the well established aromatic amine carcinogens or to other factors.

Changes in industrial practice and public health legislation in the United Kingdom have led to the abandoning of the manufacture and use of the recognized carcinogenic amines. Two problems remain. There are still numbers of workmen at risk of developing bladder cancer through previous exposure. Their disease may be detected at an early stage through exfoliative cytology. The need to avoid unnecessary anxiety by offering this procedure only to patients who are believed to have been exposed to carcinogens and to avoid premature referral on cytological evidence to urological clinics was stressed (Parkes). The discontinuing of dangerous processes in industry requires some form of monitoring if it is to be demonstrated that the incidence of the disease is indeed reduced and that new hazards are not being introduced. The Medical Advisory Service to the Department of Employment (United Kingdom) has initiated a prospective survey of workers in the rubber and electric cable-making industries in which the use of carcinogenic chemicals was thought to have ceased in 1948 (Owen).

Clemmesen advanced the view that cigarette smoking made a substantial contribution to the incidence of cancer of the bladder in many countries. In the United Kingdom, where two surveys have failed to demonstrate a convincing association between bladder cancer and cigarette smoking, he suggested that susceptible persons succumbed to carcinoma of the bronchus and lung at an earlier age than that at which bladder cancer developed.

The factors which lead to the precipitation of the remainder of the incidence of bladder cancer are not well understood. Persons who consume excessively large amounts of analgesics such as phenacetin, phenazone (antipyrine) and caffeine develop renal papillary mecrosis, renal pelvic and bladder cancer (Bengtsson). It is not known which of the three drugs is responsible for the tumours. The evidence for the

possible association between urinary schistosomiasis and tumours of the bladder in Mozambique was discussed (Macedo). It was suggested that the generally diseased state of the infested population might make them especially susceptible to carcinogenic agents.

Abnormal tryptophan metabolism has been held to be involved in the genesis of bladder cancer. The fact that 3-hydroxykynurenine and 3-hydroxyanthranilic acid, amongst other metabolites, are carcinogenic in the bladder implant tests as well as inducing leukaemia on injection into mice possibly indicates that they are carcinogenic. Bladder cancer patients in Wisconsin but not in Boston often show elevated levels of these tryptophan metabolites, a paradox which has been explained by the greater extent of occupational exposure to bladder carcinogens in the latter series (Brown). However, administration of tryptophan metabolites to mice without a pellet (Bryan) or directly to the urinary bladder through a ureto-cutaneous fistula (Kerr) have failed to induce bladder cancer. The evidence for the participation of these endogenous metabolites in bladder carcinogenesis is considered to be weak.

THE CHEMICAL INDUCTION OF BLADDER CANCER

The majority of reports dealt with the molecular structure and metabolic activation of bladder carcinogens. There is a reluctance to study the biochemical capabilities of the tissue, possibly because of the technical difficulties involved in working with small quantities of mixed populations of cells. Two agents, N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (Bryan *et al.*; Tiltmann and Friedell) and locally applied N-methyl-N-nitrosourea (Hicks *et al.*) are able to induce papillomas and carcinomas of the bladder epithelium within a short period and in the majority of the treated rats. This shows that the relatively lengthy induction periods and low incidences obtained with other chemicals are more likely to result from the low efficiency of metabolic activation than from an intrinsic resistance of the bladder epithelium to carcinogenesis.

The toxin in bracken which is responsible for haematuria and bladder cancer in cows (Pamukcu *et al.*) induces intestinal and bladder tumours in rats. Feeding bracken fern with a supplement of thiamine hydrochloride or riboflavine enhances the yield of bladder tumours. There is a lack of epidemiological evidence on the tumour incidence in the human population in areas where there is enzootic haematuria in cattle, but attempts are being made (Evans *et al.*) to determine whether the milk from bracken-fed cows is carcinogenic in other species. The chemical structure of the bracken toxin is proving difficult to elucidate. It has the properties of an unsaturated lactone, a molecular weight of 156 and molecular formula $C_7H_8O_4$.

One of the more difficult problems in unravelling the metabolic activation of bladder carcinogens lies in the need to demonstrate that the postulated intermediate is locally effective in the bladder epithelium. The technique of bladder implantation suffers from several disadvantages not the least of which is that the bladder epithelium is rendered hyperplastic and inflamed by the presence of the pellet in the bladder lumen. The observation that tissue cultures of bladder epithelium take up ^{3}H-thymidine under the influence of certain derivatives of dibutylnitrosamine was used (Bertram *et al.*) to demonstrate that the glucuronide of 4′-hydroxybutylbutylnitrosamine may be an active metabolite of both dibutylnitrosamine and 4′-hydroxybutylbutylnitrosamine. In the aromatic amine series the induction of bladder cancer in dogs after the intravesical instillation of 2-naphthylhydroxylamine but not 2-naphthylamine indicated the importance of N-hydroxylation to carcinogenic activity in this species (Radomski *et al.*) This conclusion was supported by the demonstration that the urinary output of N-hydroxylamine was greater with 4-aminobiphenyl than with 2-naphthylamine, and with the latter compound than with 1-naphthylamine. This is the order of the relative carcinogenicity of the chemicals to the dog bladder.

Not all bladder carcinogens contain substructures which are recognised to confer cancer-inducing properties. The need to understand their mechanism of action was demonstrated by the recent public controversy following the decision to prohibit the use of cyclamates. The interaction of one such chemical, 4-ethylsulphonylnaphthalene-1-sulphonamide, with the mouse bladder epithelium has been shown to be complex, involving epithelial cell loss and regeneration in the acute phase and stone formation, hyperplasia, oedema and inflammation in the chronic phase (Clayson).

A summary of the papers on clinical management of bladder cancer given at this conference will appear in the British Journal of Urology.

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Studies of the Liver Glycogen of Mice During the Growth of a Glycogen-Free and a Glycogen-Containing Subline of the Ehrlich-Lettré Ascites Tumour

C. GRANZOW* and P. BEHEIM
Institute for Experimental Cancer Research, Voss-Strasse 3, 69 Heidelberg, Germany

Abstract—*By histochemical and biochemical methods a different conduct of the liver glycogen of mice was ascertained in animals bearing a glycogen-free and a glycogen-containing subline of the Ehrlich-Lettré ascites tumour (MAT).*

During the growth of the glycogen-free MAT the liver glycogen content of the mice decreases more or less continuously to about 10% of the normal value within 10 days. During the early growth phase of the glycogen-containing MAT, however, there is an initial rapid decrease of the liver glycogen leading to almost complete exhaustion, followed by a small renewed increase.

The glycogen-containing mouse ascites tumour subline shows a decrease of its intracellular glycogen content within 48 hr after transplantation, followed by a storage of glycogen which is mainly intranuclear. The intranuclear storage process begins 3 days later than the tumour growth.

INTRODUCTION

SCHOLZ and Paweletz [1] demonstrated glycogen deposits in the nuclei of the hyperdiploid Ehrlich–Lettré Ascites Tumour (MAT). By a process of selection, Scholz [2] succeeded in separating the original mixture of glycogen-containing and glycogen-free tumour cells. A glycogen-free tumour subline could be selected as well as a subline in which cells containing glycogen dominated.

Differences between these two sublines were observed also in respect to their karyogrammes [3], some further biochemical qualities [4, 5] and their response to colchicine [6]. In liver sections, after PAS-reaction, Scholz [2] observed that after 1 week's growth of the tumours, the glycogen content of the liver of the mice inoculated with glycogen-containing tumour seemed lower than in the liver of mice inoculated with glycogen-free tumour material. It is the aim of the present investigation, to compare the behaviour of the liver- and tumour-glycogen morphologically and quantitatively during the growth of the two tumour lines.

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*Present address: Temple University, Department of Biology, Philadelphia, Pennsylvania 19122.

Reprint requests and inquiries should be addressed to Dr. P. Beheim, at the German Cancer Research Centre.

MATERIAL AND METHODS

(a) *Experimental animals*

The experiments were performed using unselected male albino mice from a conventional source with an average weight of 37·5 g. The animals were distributed at random over the experimental groups and fed with usual dried food.

(b) *Tumour sublines*

The tumour material of the glycogen-containing subline of MAT is moderately viscous and of a light brown colour. A smear of ascites cells from a 7-day grown tumour, after the PAS-reaction, is shown in Fig. 1: 80 to 95% of the tumour cells show PAS-positive deposits of different sizes. These deposits are mainly intranuclear, but are also present in the cytoplasm. Before the beginning of the experiment, this distribution of the glycogen had been regularly observed in 26 controlled passages.

The tumour material of the glycogen-free subline of MAT is more viscous and of a whitish colour. Under the microscope the nuclei of the tumour cells appear glycogen-free after the PAS-reaction. Very rarely minute PAS-positive granules appear in the cytoplasm. Often the leukocytes contain PAS-positive material. In 24 controlled passages before the beginning of the experiment, the absence of glycogen within the nuclei of the tumour cells had been regularly noted.

The transplantation of the tumours was always performed with 7-day grown ascites tumour. The transplanted dose was 0·2 ml per mouse.

(c) *Experimental procedure*

In both tumour sublines the design of the experiment was the same. For each tumour subline, 55 mice were divided into 11 equal groups. At the start of the experiment the 5 animals per group were inoculated with 5 different tumour samples from the same source. Out of the 11 experimental groups, 1 group was left uninoculated and was used as a control for the normal liver glycogen content. Every day between 9 and 10 a.m., from the 1st to the 10th day after inoculation, the 5 animals of one group were sacrificed by dislocation of the neck.

After aspiration of the available tumour volume, the liver was removed, weighed, and rapidly frozen at −70°C. After measurement of the volume, the ascites tumour was centrifuged (15 min, 4°C, 1400 g). The supernatant was rejected. The sedimented cell mass was weighed and rapidly frozen. For histochemical investigation, 2 smears were prepared from each tumour. Samples of liver and ascites cells which could not be processed immediately were stored at −25°C. Previous experiments had shown that in this case the glycogen content of the samples did not change demonstrably during storage of up to 3 weeks.

The enzymatic determination of the glycogen content was carried out with the single livers of all experimental animals. The tumour samples from the first 2 days, being very small, were pooled and an average analysis was performed. At the following days, single examination was performed with each sample of the glycogen-containing tumour and with 2 samples per day of the glycogen-free tumour.

(d) *Light miscroscopic preparation and evaluation*

After fixation with osmic vapour, the Feulgen reaction was performed on 1 smear from each tumour sample. On another smear of the tumour, the PAS-reaction was performed, after fixation with Carnoy's fluid. The liver biopsy from each experimental animal was cut into 2 parts. From one part, 10 μ thick frozen sections were prepared, fixed with osmic vapour for 20 sec, treated for 15 min with Rossman's mixture [7], and then the PAS-reaction was performed. The other segment of the liver biopsy was fixed with Carnoy's fluid and embedded in paraffin. The PAS-reaction and the Azan-staining according to Heidenhain [7] were performed on 7 μ thick serial sections.

In the tumour smears stained according to the Feulgen procedure, the mitotic index was determined. The remaining preparations were qualitatively evaluated.

(e) *Quantitative glycogen determination*

To determine the glycogen content of the material (liver or MAT-cells), the crude glycogen was isolated first according to a modification of the method elaborated by Good and co-workers [8]. The material was heated in 30% KOH (3 ml/g of tissue) for 30 min in a boiling water bath. After the solution had cooled down, 1·5 volumes of 96% was added, the mixture was again brought to the boil and then left to stand for 3 hr at room temperature. The precipitated crude glycogen was centrifuged (15 min, 4°C, 2500 g), washed twice with 96% ethanol and dried in the dessicator.

For deproteinisation the crude glycogen was dissolved in 0·5% perchloric acid. After 1 hr in the ice bath it was centrifuged (20 min, 4°C, 2500 g), and the sediment washed in 0·5 N perchloric acid. Each glycogen-containing supernatant was neutralized with a 0·5 N solution of potassium bicarbonate, and a 0·2 ml aliquot was used for the determination of glycogen. Hydrolysis was performed by the addition of 0·6 ml of 0·2 M acetate buffer pH 4·8 and 0·2 ml of amylo-α-1·4-α-1·6-glucosidase suspension to this aliquot, followed by shaking for 45 min at 40°C [9].

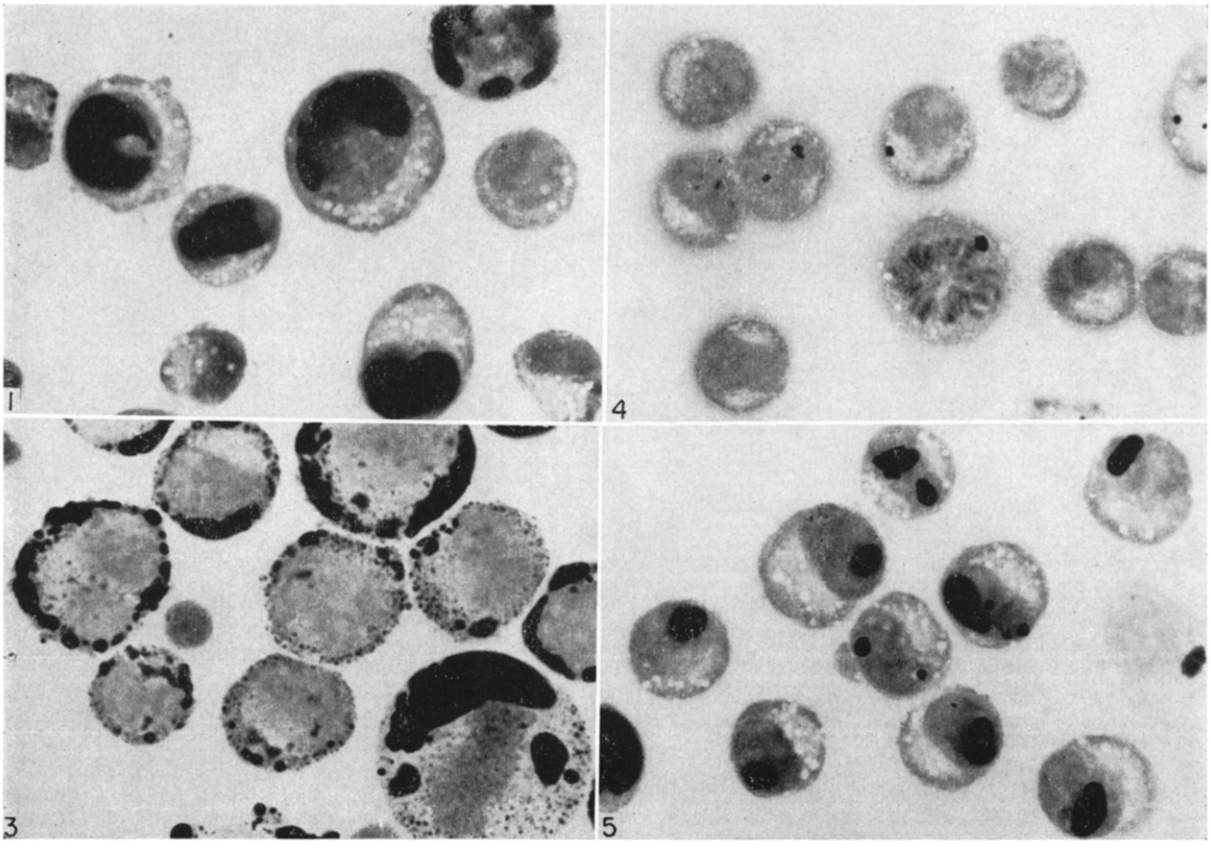

Fig. 1. *Cells of the glycogen-containing MAT 7 days after tumour transplantation. Fix.: Carnoy; stain: PAS-reaction. Magnification:* 1200 × .

Fig. 3. *Cells of the glycogen-containing MAT 24 hr after tumour transplantation. Fix.: Carnoy; stain: PAS-reaction. Magnification:* 1200 × .

Fig. 4. *Cells of the glycogen-containing MAT 72 hr after tumour transplantation. Fix.: Carnoy; stain: PAS-reaction. Magnification:* 1200 × .

Fig. 5. *Cells of the glycogen-containing MAT 6 days after tumour transplantation. Fix.: Carnoy; stain: PAS-reaction. Magnification:* 1200 × .

(to face p. 226)

The glucose thus obtained was specifically determined with hexokinase and glucose-6-phosphate-dehydrogenase [10].

Measured values which fell below 0·05% glycogen were rounded up to 0·05%, since for methodical reasons they could not be determined with accuracy.

The enzymes for hydrolysis of glycogen and for determination of the glucose were acquired from the Boehringer Mannheim Company.

RESULTS

Out of 50 mice per tumour subline, data could be obtained from 44 mice inoculated with glycogen-containing and from 45 mice inoculated with glycogen-free tumour material.

Three and 4 days after transplantation, the tumour cells showed the lowest relative glycogen content (less than 0·05% in 6 of 8 specimens). The absolute amounts of tumour glycogen, determined singly in 32 specimens, grown 3 and more days, lay between 0·14 mg (4-day grown tumour) and 66 mg (10-day grown tumour). It is a close correlation between the tumour volume and its glycogen content (Fig. 2; correlation coefficient $r = 0{\cdot}8269$, $p < 0{\cdot}01$). Under the microscope, in the smears of glycogen-containing tumour cells, intracellular variations of the amount and shifts in the arrangement of the glycogen were observed within the early phases of the tumour development. Figure 1 shows that in 7-day old tumour material, such as was used for the inoculation of

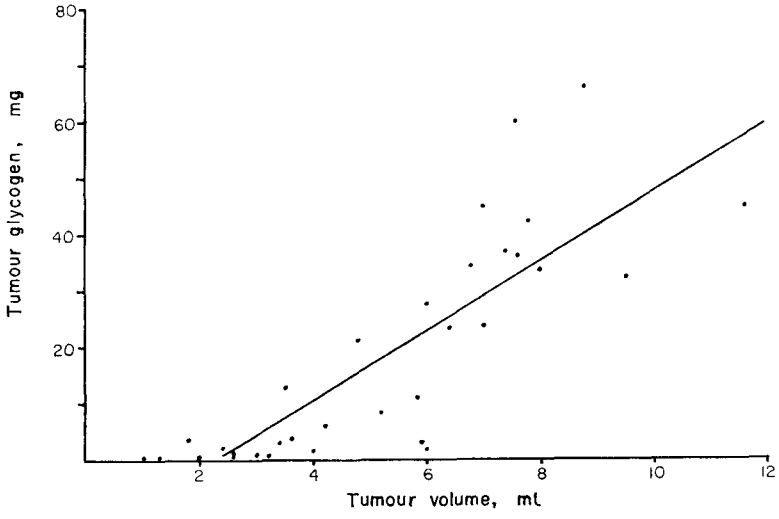

Fig. 2. Correlation between volume and glycogen content of the glycogen-containing MAT.

(a) *Growth rate of the tumours*

The mitotic index varied between 0·8 and 4·6% in the glycogen-containing tumour, and between 0·8 and 3·7% in the glycogen-free tumour. The highest values were observed in both tumours within the first 48 hr after inoculation. The average volume of the tumour specimens grown more than 2 days was $5{\cdot}2 \pm 2{\cdot}6$ ml ($n = 32$) in the glycogen-containing tumour and $5{\cdot}7 \pm 4{\cdot}0$ ml ($n = 35$) in the glycogen-free tumour.

(b) *Behaviour of the tumour glycogen*

(1) *Glycogen-containing ascites tumour.* Twenty-four hr after tumour inoculation, the amount of glycogen was 1·06% of 0·2 g tumour cells = 2·1 mg (sum of 4 pooled samples), 48 hr after inoculation 0·09% of 2·04 g tumour cells = 1·8 mg (sum of 5 pooled samples). the experimental animals, considerable amounts of PAS-positive material are present mainly in the tumour cell nuclei.

Figure 3 represents the aspect 24 hr after tumour inoculation. PAS-positive material is present almost exclusively in the cytoplasm, whereas the nuclei are completely free of it.

Figure 4 shows the phase of the smallest glycogen content of the tumour cells 72 hr after transplantation. Most of the cells are glycogen-free; but in the few cells in which glycogen can be found, it is seen as very tiny drops in the nuclei and in the cytoplasm.

With the further growth of the tumour, we found a rapid decrease in the number of glycogen-free cells in relation to the glycogen-containing cells. At first, it was frequently observed that many small glycogen deposits could be found in the same nucleus. Later, these intranuclear deposits appeared to increase in size and to fuse together. Figure 5 shows

tumour cells in the middle phase of growth, whose nuclei contain PAS-positive inclusions of various sizes. At this stage, cytoplasm only rarely shows PAS-positive material.

(2) *Glycogen-free ascites tumour.* In the glycogen-free tumour, during the earliest phase of growth minimal amounts of glycogen (in most cases below 0·05 mg) could be traced biochemically. Microscopically, PAS-positive material could be found merely within intermingled leukocytes. The tumour cells themselves were free of it.

Similarly in both kinds of tumour, the decrease of the liver glycogen is accompanied by a change of its topohistochemical pattern. In general, the disappearance begins in the centres of the liver lobules and progresses towards their periphery. When the liver glycogen content falls below 10% of the normal, only disconnected strips or islands of glycogen-containing liver cells can be found near the terminal branches of the portal vein.

In some of the mice bearing glycogen-containing tumours, essentially no glycogen could be detected in the liver by the histochemical method.

Table 1. Tumour volume and glycogen content of the liver in mice bearing glycogen-containing and glycogen-free MAT (G+ : glycogen-containing MAT, Gϕ : glycogen-free MAT).

Tumour volume (ml)	<3·0		≧3·0 < 6·0		≧6·0		Control
Kind of tumour	G+	Gϕ	G+	Gϕ	G+	Gϕ	Healthy mice
Liver glycogen (mg)	1	6	1	6	1	2	34
	1	9	1	6	1	2	38
	5	9	1	11	1	2	38
	9	14	1	11	1	3	42
	10	19	1	11	3	3	42
	17	19	1	12	3	3	43
	25	21	1	13	4	3	44
	26	31	2	14	4	4	60
	31	34	2	17	9	7	68
	38	41	2	22	12	8	74
	38	42	4	34	13	8	
	40	42	4		13	9	
	40	46			15	11	
	68	46				12	
	71	49				16	
	74	49					
	115	50					
		55					
		63					
		65					
		66					
		82					
Number	17	22	12	11	13	15	10
Mean	38·5 ± 31·0	39·0 ± 21·0	1·8 ± 1·1	14·3 ± 8·0	6·2 ± 5·4	6·2 ± 4·4	48·8 ± 13·7

(c) *Behaviour of the liver glycogen*

In Table 1, the biochemically ascertained amounts of liver glycogen are correlated to small, middle, and large tumour volumes. At volumes between 3 and 6 ml, the liver glycogen content is significantly lower in mice bearing glycogen-containing tumour than in mice bearing glycogen-free tumour.

DISCUSSION

In the spontaneously arisen new subline of the MAT, so far the glycogen nature of the nuclear inclusions had been postulated solely on the basis of histochemical and electron microscopic findings [1]. The glycogen content of the tumour cells could now be proven by means of the highly specific enzymatic method

of determination. The growth of this tumour, recognizable by the increase of its volume, progresses continuously and is characterized by a stimulated mitotic activity during the first 3 days after the transplantation. The behaviour of the glycogen content, however, is different from it. It decreases during the first 3 to 4 days of growth to a minimum near zero, resulting in a rapid drop of the relative glycogen content of the tumour cells (from 1·06 to about 0·05%).

A reversal occurs, as soon as the tumour volume exceeds about 2 to 3 ml. The following phase of growth shows a strong correlation between the volume and the glycogen content of the ascites tumour. A process of glycogen storage occurs, indicated by the continuous increase of the absolute and relative amounts of glycogen. The described phenomena are accompanied by drastic morphological changes of the tumour cells. Twenty-four hr after the inoculation of cells whose nuclei exhibit a massive quantity of glycogen, the nuclei are completely glycogen-free. High amounts of glycogen, however, are seen within the cytoplasm.

Forty-eight hr later, the tumour cells appear essentially glycogen-free. In a few cells, the cytoplasm still contains minute PAS-positive inclusions, and in a few other cells, frequently multilocular traces of glycogen reappear within the nuclei. Later, these inclusions increase continuously. All together these intraperitoneally growing tumour cells were found capable of intranuclear storage of glycogen. Probably due to the changed environmental conditions, a shift of glycogen deposits occurs from the nucleus to the cytoplasm immediately after tumour transplantation. The intranuclear accumulation of tumour glycogen is interrupted during the first phase of development and starts 3 to 4 days later than the tumour growth.

As Young and coworkers [11] using the Sarcoma 180 demonstrated, tumour bearing mice exhibit an impairment in their ability to store liver glycogen. In both kinds of the MAT, which we have investigated, the growth of the tumours is accompanied by a decreasing liver glycogen content of the tumour bearing animals. Histotopochemically, the disappearance of the glycogen progresses regularly from the centre to the periportal periphery of the liver lobules. The quantitative determination showed a characteristic difference between mice bearing glycogen-free and glycogen-containing MAT. In correlation to the increase in tumour volume, the glycogen content of the mouse liver decreases more or less continuously in the glycogen-free MAT. In contrast in animals with glycogen-containing MAT, an initial rapid decrease leads directly to an almost absolute depletion of the liver glycogen for a considerable phase of the tumour growth. Finally, however, the situation is the same as that of the glycogen-free MAT. In the middle phase of tumour growth, therefore, the livers of mice bearing glycogen-storing MAT contain far lower amounts of glycogen than those of the mice with glycogen-free MAT. The renewed intranuclear glycogen storage of the tumour cells described above passes through its first stages within this phase of tumour development. During this phase, the glycogen-containing MAT probably exerts a far greater strain on the carbohydrate metabolism of the host than the glycogen-free MAT. In later phases of tumour growth no difference concerning the liver glycogen content exists between the two kinds of MAT investigated.

These facts suggest a special tumour-host-relationship in the new glycogen-storing subline of the MAT.

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A New Malignant Lymphoma Induced in the Syrian Hamster

ABRAHAM KARPAS and ALBERT SAMSO

Department of Medicine, University of Cambridge, and Service des Virus, Institut Pasteur, Paris

Abstract—*Two Syrian hamsters of different litters developed malignant lymphoma after inoculation with* in vitro *cultured embryonic hamster cells infected with equine herpes* 3 (EH_3) *virus. A causal relationship between* EH_3 *virus and the lymphoma could not be proved. However, the tumour could be induced in new-born hamsters with subcellular material. This suggested a viral aetiology. The lymphoma differed from earlier-studied hamster tumours in its karyotype, its very high metastatic rate as well as its frequent* (71%) *cardiac metastases. Adult hamsters which were inoculated with the tumour suspension developed complement fixing antibodies to a heat-labile tumour antigen. Preliminary experiments on the possible mode of natural transmission suggest that this malignancy can be transmitted vertically as well as horizontally.*

INTRODUCTION

THE OCCURRENCE of spontaneous lymphoma in the Syrian hamster has been recorded occasionally. Several attempts to transmit a hamster lymphoma with cell-free tumour tissue have failed [1–3]. However, Brindley and Banfield [2] have shown that uninoculated hamsters, caged with tumour-bearing ones, did develop a lymphoma. They also showed that the disease was transmitted even when hamsters were separated from each other by a screen. Graffi *et al.* [4] working with a hamster colony free from spontaneous leukaemia, could induce a lymphoma in the new-born with a cell-free filtrate obtained from hamster skin tumours. They detected a Papova virus in the skin tumours by electron microscopy. Further passage of this tumour material yielded only C-type (RNA) particles. Stenback *et al.* [5] reported that a malignant lymphoma of the hamster contained many budding C-type particles which could also be found in the blood plasma of tumour-bearing animals. Toth [6] reported that injection with a carcinogen induced a lymphoma in the Syrian hamster.

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This report deals with the study of a malignant lymphoma which appeared in two Syrian hamsters of different litters, after inoculation with *in vitro* cultured embryonic hamster cells infected with equine herpes 3 (EH_3) virus [7]. Although it appears that this lymphoma is histologically similar to earlier-reported hamster lymphomas, the pathology of the disease, the rate of metastasis, the organs in which metastases occur and the karyotype distinguish this lymphoma from hamster lymphomas studied previously. In view of the fact that the role of several herpes viruses in the induction of, or association with, malignancy has been established recently, an attempt was made to learn whether EH_3 virus or other viruses are associated with the induction of this tumour. Successful induction experiments with subcellular tumour material suggest that this lymphoma is virus-induced and that it may be transmitted naturally, although a virus has not been isolated so far.

MATERIAL AND METHOD

Tissue cultures and media

(1) Hamster embryo (HE) cell cultures were prepared from embryos close to term, using the standard trypsinization technique.

(2) Primary rabbit kidney (RK) cell cultures were prepared by trypsinization of kidneys obtained from young rabbits (approximately one month old).

(3) A kidney with a large metastatic tumour from a subcutaneously (s.c.) inoculated hamster was trypsinized and the cells grown in culture.

(4) Suspension cultures of tumour lymphoblasts were obtained by mincing soft tumour tissues with sharp scissors.

"Basal" Eagle's medium (BME) supplemented with 10% calf serum, 200 units per ml of penicillin and 200 μg per ml of streptomycin was used.

Tissue culture inoculation

(1) Hamster embryo (HE) cell cultures were infected with EH_3 virus [8] and observed for cytological changes.

(2) RK cultures were infected with tumour extract as well as with *in vitro*-cultured lymphoblasts to see whether an infective EH_3 virus could be recovered from the hamster lymphoma.

(3) Hamster embryo cell cultures were infected with tumour extracts and screened for cytological changes. Infected and non-infected cultures were observed for at least one month and when cytological changes were suspected the cultures were fixed and stained with haematoxylin and eosin (H. & E.).

Inoculation of new born hamsters with hamster embryo cells

Detailed description of the inoculation of HE cells, infected and non-infected with EH_3 virus, into new-born litters of Syrian hamsters is given in another paper [9]. Briefly, the third passage of HE cells grown in a Roux bottle were infected with EH_3 virus at an approximate ratio of one virus particle per ten cells. Four days later, the cells were trypsinized and seeded into two Roux bottles. Eight days thereafter the cells of one bottle (2nd pass after EH_3 infection) were harvested and injected s.c. into one litter of 10 new-born hamsters. The other bottle was trypsinized and cells seeded into two Roux bottles. Seven days thereafter, the cells of one bottle (3rd pass after EH_3 viral infection) were harvested and inoculated into 21 new-born hamsters of three litters.

Serial transplantation of tumour tissue

A 20% tumour suspension in phosphate buffer saline (PBS) was prepared from non-necrotic tissues which were minced finely. New-born hamsters were inoculated with 0·1 to 0·2 ml, while older animals were inoculated with 0·5 ml. In most cases the animals were inoculated s.c., in the back. On several occasions hamsters were inoculated intraperitoneally (i.p.). Serial transplantation of the tumour was carried out at irregular intervals.

Preparation of tumour extract

A 40% tumour suspension was prepared in BME. The tumour tissue was first minced and then homogenized in an electric homogenizer. The homogenate was frozen and thawed (−70°C to 30°C) three times. The suspension obtained was centrifuged in a refrigerated centrifuge for 30 min at 2500 rev/min. The upper two thirds of the supernatant was collected and used for inoculation of new-born hamsters as well as for inoculation of normal primary cells of hamster embryos.

Inoculation of hamsters with sub-cellular material

(1) Three litters of new-born hamsters were inoculated s.c. with 10^4 infectious EH_3 virus particles grown in RK cells.

(2) Three litters of new-born hamsters were inoculated i.p. with 0·2 ml of the cell free tumour extract.

The surviving hamsters were observed for 1 year.

Neutralization tests

Samples of sera from 4 hamsters that had been carrying the tumour for over 2 months were inactivated separately at 56°C. for 30 min. Equal amounts of either undiluted or diluted (1 : 5) sera were mixed with a suspension of EH_3 virus containing 10^3 $TCID_{50}$/ml and incubated in a 37°C. water bath for 1 hr. Following incubation, the virus–serum mixture was inoculated into RK cells which were then kept for 2 more weeks at 37°C.

Fluorescent antibody staining

Cultures of EH_3-virus infected and non-infected RK cells grown on coverslips were used with serum obtained from tumour-carrying hamsters. The indirect staining method of Weller and Coons [10] was employed.

Preparation of tumour antigens for complement fixation tests (CFT)

A 20% homogenate of tumour tissue was prepared by homogenizing the tissue in BME. This homogenate was frozen and thawed 4 times (−70°C to 25°C) and centrifuged at 2500 rev/min for 10 min. The supernatant was used

as the tumour antigen in the CFT. Three different preparations of tumour antigen were tested, using the same technique:

(1) Tumour tissue obtained from adult hamsters, which had been inoculated with suspension of tumour tissue when adults.

(2) Tumour tissues obtained from 2-week old hamsters which were inoculated when newborn with suspension of tumour tissue.

(3) Same tumour antigen which was used in preparation 2 (from 2-week old hamsters), but heated at 56°C for 30 min.

Prior to the CFT, the antigen preparations were kept at −70°C.

Hamster sera for complement fixation tests

Sera were obtained from numerous tumour-carrying hamsters at different time intervals after inoculation with tumour suspension. All sera were inactivated at 60°C for 30 min and stored at −20°C.

Complement fixation tests

The standard technique was employed in which 0·1 ml of serum, antigen and complement (1·5 units) were incubated overnight at + 4°C. This was followed by the addition of 0·2 ml of 1% sensitized sheep RBC, to give a total volume of 0·5 ml.

The anti-complementary activity of the antigens and sera was determined prior to the tests.

Preliminary investigations into vertical or horizontal transmission

1. *Vertical transmission.* Four females were inoculated s.c. with 1 ml of 20% tumour suspension. Three days later they were introduced individually to different un-inoculated males. Approximately three weeks later the females gave birth to litters of various numbers. During pregnancy tumours developed at the site of the inoculation. The offspring of each female were kept together and interbred. Those which survived the first few weeks developed normally. Pregnant females of the F_1 generation were separated and their offspring, the F_2 generation, were watched during their growth.

2. *Horizontal transmission.* The new-born litters of four normal females were inoculated with tumour suspension and were left with their mothers. The mothers were not inoculated and observed for horizontal transmission over a period of 4 months. If they would develop a lymphoma following contact with their inoculated litters, it would suggest a transmission of the causative agent from the baby hamster to its mother.

RESULTS

Following the initial inoculation of the *in vitro*-cultured hamster embryo cells with EH_3 virus, foci of cytopathogenic effect (CPE) developed. After the medium was changed the foci of cell degeneration disappeared and a confluent cell sheet appeared all over the glass surface. The examination of stained preparations of the EH_3 virus infected and non-infected primary hamster embryo cells revealed that non-infected cells were of uniform morphology, but the cell sheet was not a monolayer, rather a multilayer. The EH_3 viral infected cells sheet was not so dense as the non-infected cultures. The morphology of many of the cells was altered but only rarely could a herpes-type intranuclear inclusion be found. The most prominent changes in the infected cultures concerned the nuclei; these showed a wide range of morphological deviation from those in the control preparations [7]. In spite of the altered morphology the cell culture remained viable and continued to grow following trypsinization and sub-culturing. It was suspected that cellular transformation may have been induced by the EH_3 virus and, therefore, newborn hamsters were inoculated with those cells. During the eighth month it was noticed that one of the hamsters from the litter of 10 which had been inoculated with the second passage of EH_3 virus infected HE cells (of which all survived), developed a s.c. growth. This growth continued to increase in size in the following weeks. The animal was killed and sections of the tumour tissue indicated that it was a lymphosarcoma (Fig. 1). The tumour was also used for the preparation of a suspension which was injected into hamsters of various age. From the 21 hamsters (of 3 litters) which were inoculated with the third passage of EH_3 virus infected HE cells, only 11 survived the immediate post-inoculation period. Among those 11 animals, a growth identical to the lymphoma which developed in the other hamster, was detected in one animal on the 22nd month following inoculation. None of the other animals inoculated developed a malignant growth, nor did any of the hamsters which were inoculated with the non-infected HE cells.

Inoculation of hamsters with tumour suspension

Animals inoculated s.c. or i.p. with suspensions of tumour cells developed lymphomas.

The survival time was related to the route of inoculation, the age of the animal at the time of inoculation, the amount of tumour material injected, as well as to the localization of metastatic tumours. The survival time of adult hamsters inoculated s.c. was of the order of 2 to 3 months in most cases.

Systematic post-mortem examination of a group of 32 hamsters which were inoculated s.c. with 0·5 ml of 20% tumour suspension revealed that metastases occurred in all animals except one. The frequency of affected organs was as follows: viscera 84%, heart 71%, thymus 67%, lungs 65%, kidneys 50%, liver 16%, eyes 13%, ovary 13%, axillary lymph nodes 13%. Metastases were also recorded in the inguinal lymph nodes, testicle, bladder, and a uterine horn. In spite of the fact that all hamsters were inoculated with the same amount of tumour material there was a remarkable difference in the size of the tumour developed at the site of inoculation. The size of the tumour at the site of inoculation varied considerably and this was unrelated to the extent of metastases in other organs. The frequent cardiac involvement was striking, detected macroscopically in 71% of the animals (Fig. 2). The heart muscle of many hamsters was replaced to a large extent by tumour tissue. In some hearts the myocardium was atrophic, with tumour masses even in the inter-ventricular septum. The direct cause of death in many hamsters could be attributed to heart failure. Microscopically the spleen always appeared normal in size, but histological examination revealed also metastatic foci in this organ.

Inoculation of hamsters with sub-cellular material

The 28 new-born from three litters were inoculated i.p. with supernatant of tumour extract; 14 were cannibalized or died of unknown causes while 14 survived (for over 1 month). Two months after the inoculation two hamsters showed a swollen abdomen and an abnormal growth was palpated. After a further 2 weeks the two hamsters were killed and post-mortem examination revealed tumour tissue in the abdomen with metastases in various organs. In a third hamster, an abdominal tumour could be detected 3 months following inoculation. Histopathological examination of tumour material from these three hamsters showed that the tumour was a typical lymphoma.

None of the new-born hamsters which were inoculated with EH_3 virus alone developed a tumour within 1 year of observation.

Inoculation of tissue cultures with tumour material

When RK cells were infected with tumour cells they had not undergone morphological transformation nor could infective EH_3 viral particles be recovered.

However, some of the hamster embryo cells which were infected with the tumour extract developed morphologically detectable cellular changes. Cytoplasmic vacuoles appeared 10 days after the inoculation in some cells and during the following days there was a slight increase in the number of affected cells (Fig. 3). At first only few cells seemed to be affected in one area but the foci increased in size during incubation. In stained preparations it could be seen that the cytological changes in some of the hamster embryo cells affected especially the nucleus (Fig. 4). There was great variation in nuclear size and chromatin pattern. Giant nuclei with bizarre shape could be found. Some of the cells were multi-nucleated. This was in sharp contrast to the non-infected HE cells which appeared to be fibroblastic (Fig. 5), with rather homogeneous appearance. Seven weeks after the initial infection there were foci of clustered rounded cells separating themselves from the cell sheet, and appearing as rounded cells in the fluid. They continued to grow in suspension and resembled lymphoblast cultures as grown from the original tumour tissue (Fig. 6).

Cytology of trypsinized cultures from lymphomatous hamster kidney

Initially the purpose of this study was to learn whether EH_3 virus could be recovered from trypsinized kidney cells. Several herpes viruses had been recovered previously as latent agents from apparently clinically normal animals by culturing trypsinized kidney cells; this procedure could unmask a latent EH virus. During the first 6 weeks EH_3 virus could not be recovered nor could intranuclear inclusion bodies be detected in any of the kidney cells grown *in vitro*. Only after several weeks was a limited CPE detected in the cell sheets with necrotic cells coming off. Stained cultures revealed marked cytological abnormalities affecting particularly the nuclei which appeared in many cases pleomorphic; in some cells the cytoplasm was also vacuolated (Figs. 7 and 8). It was of interest to note that the cellular abnormalities in the cells derived from a tumour-bearing hamster showed the same changes as those observed in the hamster embryo cells which were secondarily infected *in vitro* with lymphoma extract.

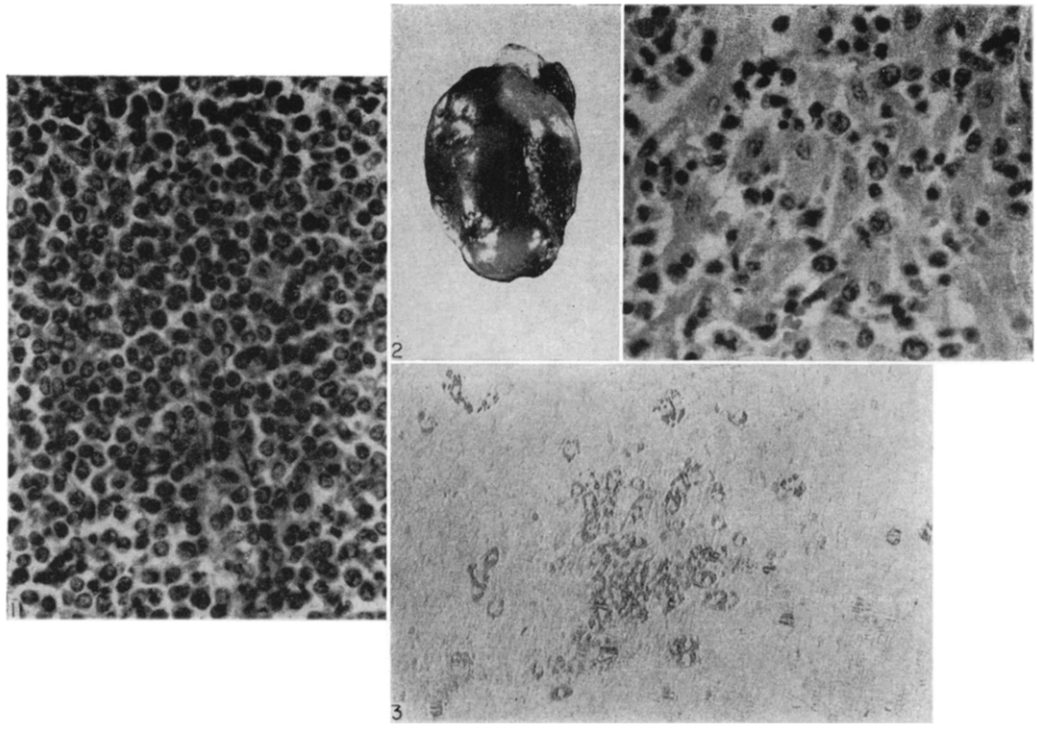

Fig. 1. *Cross-section of the lymphoma tissue in the kidney* (× 480).

Fig. 2. (a) *The heart of a hamster which was inoculated s.c. with a suspension of tumour cells at the age of one month. Six weeks later the hamsters were killed. It shows the massive metastasis in the cardiac musculature.*

(*b*) *Higher magnification of invading tumour cells between cardiac muscle fibres.*

Fig. 3. *Living secondary hamster embryo cell culture which developed cellular changes following infection with the tumour extract.*

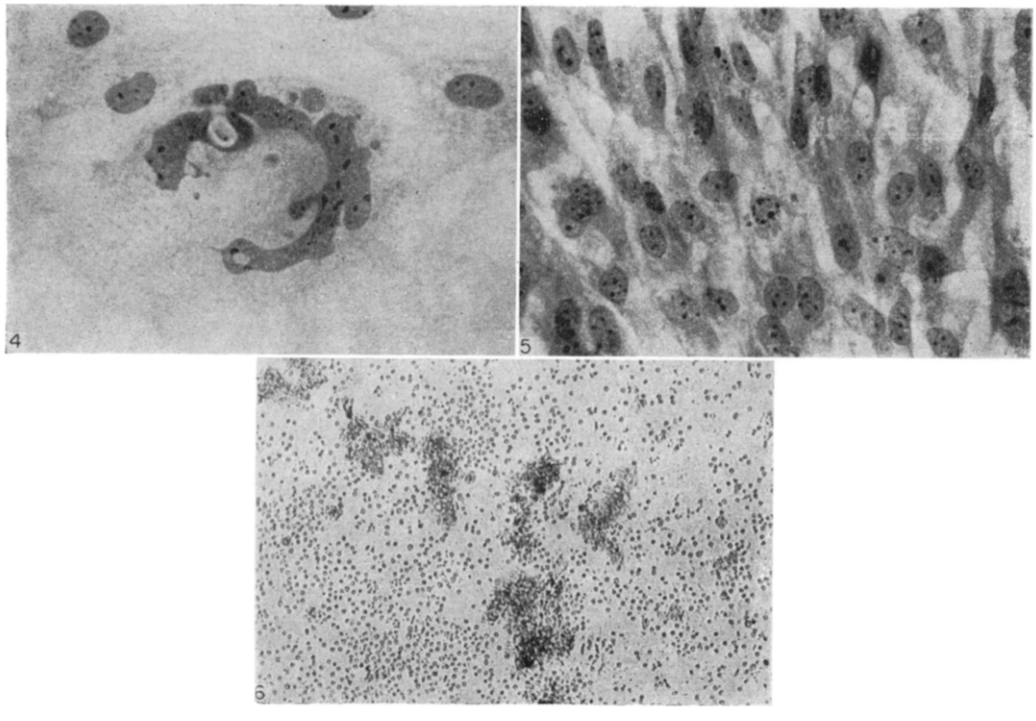

Fig. 4. *Stained hamster embryo cells which were infected with lymphoma extract. These cytological changes affecting mainly the nuclei occurred only in infected cultures* (*haematoxylin–eosin*).

Fig. 5. *Non-infected hamster embryo cell culture. The cells appear to be normal, rather uniform and grow as a monolayer* (*haematoxylin–eosin*).

Fig. 6. *Suspension of free lymphoblast-like cells which grew out of hamster embryo cells infected with tumour extract. These cells continue to grow either as individual cells or in clusters when separated from the culture.*

(to face p. 234)

Neutralisation and fluorescent antibody tests

None of the sera obtained from tumour carrying hamsters neutralized the infectiousness of EH_3 virus, nor could any difference be detected in the amount of fluorescence of RK cells treated with sera from tumour-carrying hamsters, whether the cells had been infected with EH_3 virus or not.

Complement fixation tests

One of the antigens was prepared from tumours within 2 weeks after inoculation into new-born hamsters. This antigen was the only one that reacted with sera obtained from tumour-carrying hamsters; it was inactivated by heating (Table 1). Antigen prepared from

Table 1. *Complement fixation test of sera obtained from tumour-carrying hamsters*

Hamster No.	Age at time of tumour inoculation	Days after inoculation	Titre of CF antibodies to tumour antigen	
			Unheated	Heated
109	Adult	113	< 1 : 5	< 1 : 5
110	17 days	120	1 : 10	< 1 : 5
111	Adult	113	1 : 20	< 1 : 5
2101	21 days	81	< 1 : 5	< 1 : 5
2102	21 days	81	1 : 5	< 1 : 5
2103	21 days	87	< 1 : 5	< 1 : 5
2106	21 days	87	1 : 10	< 1 : 5
2109	21 days	70	1 : 10	< 1 : 5
2652	21 days	32	1 : 20	< 1 : 5
2659	21 days	35	1 : 5	< 1 : 5

The complement-fixing antigen was prepared from tumours of new-born hamsters. The antibody carrying sera were obtained from tumour-bearing hamsters at varying stages (range 32 to 120 days). None of the positive sera reacted with tumour antigen prepared from adult hamsters.

tumour tissue from adult hamsters did not react with any of the hamster sera. The development of complement-fixing antibodies to the tumour antigen differs between the individual hamsters but was not always related to the duration of tumour-carrying period (Table 1).

The sera obtained from 10 hamsters which were inoculated when new-born with tumour suspension and lived for 1 to 2 months before being bled did not react with the tumour antigen. This may suggest a state of immunotolerance due to the introduction of the antigen before the animals became immuno-competent.

There was no relationship between the development of antibody, the size of the tumour and the rate of metastatic spread.

Transmission studies

Vertical. The F_1 generation of hamsters which were born to s.c. tumour inoculated females were observed for 3 months. During this period, no abnormal growth could be observed in the 13 hamsters that survived. Of the 13 hamsters, 7 were female; these were bred with their litter mates and gave birth to the F_2 generation. There were a total of 34 offspring in the F_2 generation, from 6 mothers. All F_1 and F_2 hamsters were observed for a further 3 months. The only litter of the F_2 generation that showed lymphomas were the offspring of hamster number 2663.

From the litter of hamster number 2663, 4 offspring survived for over 2 weeks. At 3 weeks of age, one (No. 2669) developed a small s.c. growth which increased in size during the following 6 weeks to approximately 24 cm^3. The animal was therefore killed and its blood collected. Post-mortem examination revealed that in addition to the s.c. tumour, large metastases were found in the viscera. Histopathologically, the tumour was a lymphoma. Another litter mate (No. 2672) died at the age of $3\frac{1}{2}$ months with visceral lymphoma. When 10 normal hamsters were inoculated with a suspension of tumour cells prepared from hamster 2669 all developed a lymphoma with a similar pattern of metastases frequently involving the heart.

Horizontal transmission. The litters of 4 normal female hamsters were inoculated with tumour tissue within 1 to 2 days after birth and were observed for 3 months. None of the mothers was inoculated, but a small s.c. tumour (0·03 cm^3) appeared in one of them (No. 2651), 65 days after the litter had been inoculated. The tumour increased in size during the following weeks. Seventy-two days after the first litter she gave birth to a second litter of which two survived. One developed normally while in the other hamster (No. 2652) a very large fascial tumour became apparent at 3 months of age and increased in size during the following month. The tumour tissue infiltrated the left upper jaw and the mandibular and cervical lymph nodes were enlarged. At 4 months of age, post-mortem examination of this animal revealed multiple metastases in lungs, right kidney and mediastinum. The tumour masses were attached to the thoracic vertebrae. Its mother (No. 2651) died 40 days after the initial observation of the s.c. tumour and post-mortem examination revealed large tumours in the heart and metastases in the liver. The tumour carrying suckling litter of this hamster may have transmitted the causative agent (horizontally) to the

mother (No. 2651) which had not been inoculated. Its offspring (No. 2652) of the second litter, which had not been inoculated, may have contracted the agent from the tumour-bearing mother (vertical transmission).

DISCUSSION

Biology of the tumour

The initial observations that led to the present investigation were made on cultured HE cells infected with EH_3 virus. Under these conditions the morphology of some of the cells was changed, when compared with non-infected cultures. Furthermore the altered cells continued to multiply and were passaged four times in spite of these changes. Thus, a possible virus-induced transformation *in vitro* was suspected. Recent knowledge about the possible role of herpes viruses in the induction of malignancy adds interest to these changes that occur *in vitro*. Because of the differences between this tumour and hamster lymphomas studied previously, the question arises whether this lymphoma is in any way related to EH_3 virus, and whether it carries detectable "fingerprints" of its genome. For this reason it was essential to know more about the pathology of the disease and the possible modes of transmission. So far no unequivocal proof for the association of EH_3 virus and the hamster lymphoma has been established, nor did the preliminary electron microscopic examination of tumour tissue reveal any detectable viral particle (unpublished observation). The possibility that EH_3 virus may cause an early expression of oncogenesis by activating another repressed viral gene in the infected hamster embryo cells cannot however be excluded.

Histologically, it is hard to distinguish this tumour from other reported hamster lymphomas, but it differs by its very high metastatic rate involving an unusual wide range of organs. This is in contrast to the hamster lymphoma studied by Greene and Harvey [11]. They found metastases in less than 10% of the inoculated animals. Of particular interest in the present study was the high frequency of cardiac involvement by which this tumour is characterized. An incidence of 71% for cardiac involvement has not been described before for any hamster lymphoma.

A further difference between this and an earlier-studied hamster lymphoma is found in their karyotype. While cytogenetic analysis has shown that our tumour cells contained only 39 chromosomes and is therefore hypodiploid the tumour cells of the other studied hamster lymphoma carry 51 chromosomes [12]. The normal chromosomal component of the Syrian hamster is 44 chromosomes.

Serology

It has been established that hamsters which are inoculated with a "foreign" DNA virus, like adeno and SV–40, develop tumours. Under these circumstances, complement-fixing antibodies to early heat-labile viral antigen can be found in their serum. This antigen can be detected in the infected cells before the appearance of cytopathogenic effects and before the formation of mature virions. The antigen is also found in infected tissues during the lytic phase. However, such early detection in the case of herpes viruses presents great difficulties, as their whole growth cycle is of the order of 5 hr [13] in contrast to 16 hr for adeno viruses [14] and 20 hr for SV–40 virus [15]. In the case of EH_3 virus, with its very small generation size the detection of an early antigen was not feasible. Therefore it could not be decided whether the heat-labile CF antigen found in the hamster lymphoma tissue may have been coded by an incomplete, earlier-incorporated EH_3 virion. It was interesting to note that many of the adult hamsters which were inoculated with tumour tissue developed antibodies which fixed complement with the heat-labile tumour antigen. The observation that only tumour antigen prepared from new-born hamsters reacted with sera from tumour-carrying older hamsters, suggests that the introduction of the tumour antigen into an immunologically immature animal did not result in an immune response in spite of tumour development. The introduction of tumour antigen into adult animals may have resulted in antigen–antibody complex at the cellular level of the tumour, and this may account for the high anti-complementary activity and loss of complement fixation of antigen prepared from tumours developed in adult animals.

Transmission

The limited studies on the mode of transmission of our lymphoma indicated that cell-free material could induce the malignancy in about 20% of the animals inoculated. The susceptibility of hamsters to inoculation with cell-free tumour material, depends probably on the genetic resistance or susceptibility of the strain of hamsters used, as has been suggested by Graffi *et al.* [4]. The genetic "resistance" may not be absolute; the concentration of infectious virus in the inoculum may have been too low to cause a higher percentage of lymphomas in the inoculated animals.

The observations reported in this study suggest that both vertical and horizontal transmission may take place. While none of the F_1 generation of hamsters born to tumour-carrying females and normal males had early tumours, in the F_2 generation one hamster developed a sizeable subcutaneous lymphoma by the third week. A litter mate of this hamster died at the age of 3 months with a similar tumour. Horizontal transmission from a tumour-carrying litter to the mother probably occurred in hamster No. 2651. It developed a "spontaneous" lymphoma which could be detected grossly 2 months after its litter had been inoculated with tumour tissue. The same hamster (No. 2651) gave birth to a second litter while carrying the tumour; of the 2 surviving young, one developed normally, but the other (No. 2652) died at the age of $3\frac{1}{2}$ months with multiple metastases of a malignant lymphoma. The earlier reports dealing with spontaneous lymphoma of the Syrian hamster indicated that the original tumour material was obtained from old animals over 2 years of age [2, 3, 11, 16]. This is in contrast to the earlier appearance of the tumour in the present study. Since all Syrian hamsters in laboratories are derived from two females and one male [17] the animals are inbred to a certain degree but obviously their susceptibility to tumour induction varies greatly.

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Respiration and Glycolysis in Relation to Cell Population Kinetics in Mouse Epidermis Treated with Carcinogenic or Irritant Substances

With Special Reference to the Crabtree and Pasteur Effects

OLE DIDRIK LAERUM

The Institute for General and Experimental Pathology, University of Oslo, Rikshospitalet, Oslo* 1, *Norway*

Abstract—*The changes in epidermal respiration and glycolysis have been investigated in hairless mice after a single application of the strong carcinogen 20-methylcholanthrene. The results have been compared to similar changes after a single application of cantharidin, a strong irritant, which gives a comparable degree of cellular damage with subsequent proliferation and hyperplasia. Measurements have also been performed with the solvent, benzene, which acts as a weak irritant to the skin.*

The alterations could be classified into 4 different phases:

(*1*) *A* toxic *phase during the first 4 hr after application where both compounds caused profound cellular damage with depression of both respiration of glycolysis.*

(*2*) *An* intermediary *phase from 4 to 24 hr after the treatment where most of the resulting cell loss occurs. A temporary increase of respiration and glycolysis at this stage was found to be solely due to the solvent. Later the solvent did not interfere with the results.*

(*3*) *A* growth *phase from 34 to 48 hr, characterized by an explosive proliferation in the epidermal basal cell layer. Isolated basal cells from MCA treated skin exerted a low respiration, while a normal respiration was seen after cantharidin treatment. In both cases the glycolysis of the skin was very high.*

(*4*) *A* hyperplastic *phase from 2 to 21 days after painting, where the epidermal cells had very low respiration and glycolysis.*

The main conclusions of these studies are:

(*1*) *The quantitative metabolic alterations after treatment with carcinogenic as well as non-carcinogenic irritants are due to changes both within each single cell and changes in the composition of the tissue due to altered cell population kinetics. The interpretation of metabolic studies is therefore impossible without correlating the data to simultaneous cell population studies.*

(*2*) *The Crabtree effect, which normally is of a magnitude of 10% in hairless mouse epidermis, is rapidly changing in the different phases after treatment. It is temporarily increased by the solvent. During high proliferative activity the Crabtree effect is very low, indicating that it is not related to the proliferative process. During hyperplasia, the newly formed, young cells, despite their low respiration, exert a very high Crabtree effect, which after MCA treatment is nearly 60 %.*

(*3*) *The Pasteur effect is only slightly altered in the different phases after treatment. It is moderately reduced after MCA treatment.*

(*4*) *It is suggested that the low respiration and high glycolysis during high proliferative activity after MCA treatment may have some relation to carcinogenesis.*

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*Head: Olav Hilmar Iverson.

DESPITE intensive studies no common agreement has been reached about the alterations of the energy metabolism in cancer tissue as compared to normal tissue. The problem has certainly not been lack of appropriate biochemical methods or advanced metabolic studies at the molecular level. Such studies have, however, often been performed with little or no scope to the complexity of cancer tissue, including the different kinds of tissues and the dynamics of the cell populations within the same tumour.

The purpose of the present experiments was to perform a systematic study of the relationship between alterations in some metabolic parameters and in the cell population kinetics of hairless mice during the first 10 days after a single application of the strong carcinogen 20-methylcholanthrene (MCA). A similar series of experiments was performed after application of a single dose of cantharidin which gave the same degree of cell loss and subsequent hyperplasia of epidermis, but with no carcinogenic effect.

In cancer cells *in vitro* addition of glucose causes a pronounced decrease of the respiratory rate, known as the Crabtree effect [1]. This is considered a prominent feature of the so-called "cancer metabolism", although exceptions occur, and a small Crabtree effect is also present in normal tissues [2]. It was therefore of interest to see if the Crabtree effect and also the Pasteur effect (inhibition of lactic acid production by oxygen) in the epidermis were altered during such treatment, and if these changes were possibly related to the carcinogenic process.

When presenting the results some comparisons to known changes in the cell population kinetics are introduced. This was necessary for a logical presentation of the results.

MATERIALS AND METHODS

Hairless mice (hr/hr) 70 to 200 days old were used, males and females in equal numbers. They were kept on a standard diet and given continuous water supply. The epidermal histology and normal cell population kinetics have been described by several authors [3–6].

The mice were given a single application on the right side of the back skin with the strong carcinogen 20-MCA, 1% solution in benzene and the strong irritant cantharidin, 1/16 and 1/32% solutions in benzene. A similar control group was also painted with benzene alone. The compounds were applied by means of a pair of forceps [7, 8] where the painted area is limited by a square metal frame 10 × 20 mm. The mouse is fixed by the forceps when the solution is applied and until the solvent has evaporated. By this method a constant dose is applied to a constant area, but the distribution within this area may vary. The animals were killed by neck fracture, the skins flayed off, and surface specimens were cut with a Castroviejo electrokeratotome [9]. The cutting depth was 0·2 mm, giving 8 mm broad specimens consisting of the epidermis and the upper half of the dermis. These specimens are in the following referred to as "skin pieces". The skin removed covered exactly the painted area. A normal control specimen was cut from the unpainted side of the animal. Histological controls were routinely taken from the remaining skin to ensure that all the epidermis had been removed. Specimens of pure dermis were obtained after the epithelium had been removed from skin pieces by trypsin treatment (see below). Oxygen consumption of skin pieces and pieces of dermis only was measured by ordinary Warburg technique using a Warburg constant volume respirometer (B. Braun, Melsungen, Germany) in air atmosphere. The skin pieces were incubated for 1 hr floating on the surface of Krebs–Ringer phosphate medium [10] with streptomycin 100 μg/ml. To avoid precipitates of calcium phosphates only one half of the suggested amount of calcium was used.

Lactic acid production was determined by an LDH enzymatic method using a reaction kit (Boehringer and Soehne,Mannheim,Germany). The lactic acid produced was estimated in the medium on which the skin pieces had been floating during 2 hr incubation in the Warburg apparatus; aerobically in air, and anaerobically after gassing the flasks with pure nitrogen. The medium then contained 1 mg/ml glucose. Aerobic lactic acid production and oxygen consumption with glucose were measured simultaneously.

The oxygen uptake was expressed as QO_2 or μl O_2 per mg of dry tissue per hr. The lactic acid production was expressed as μg lactic acid per mg of dry tissue per hr.

To study the respiration of epidermal single cell suspensions a separation method was used [11]. The method consists of trypsin and collagenase treatment and mechanical scraping and gives nearly pure suspensions of basal and differentiating cells respectively [12] (see Fig.1). The oxygen consumption of the two fractions was measured by an oxygraphic technique during 10 min incubation at 37°C, using a pH-meter with polarographic oxygen sensor (Beckman Instr., Inc., Fullerton, California), and was also here expressed by QO_2 or μl O_2 per mg of dry weight per hr.

The separation procedure gave basal and

A

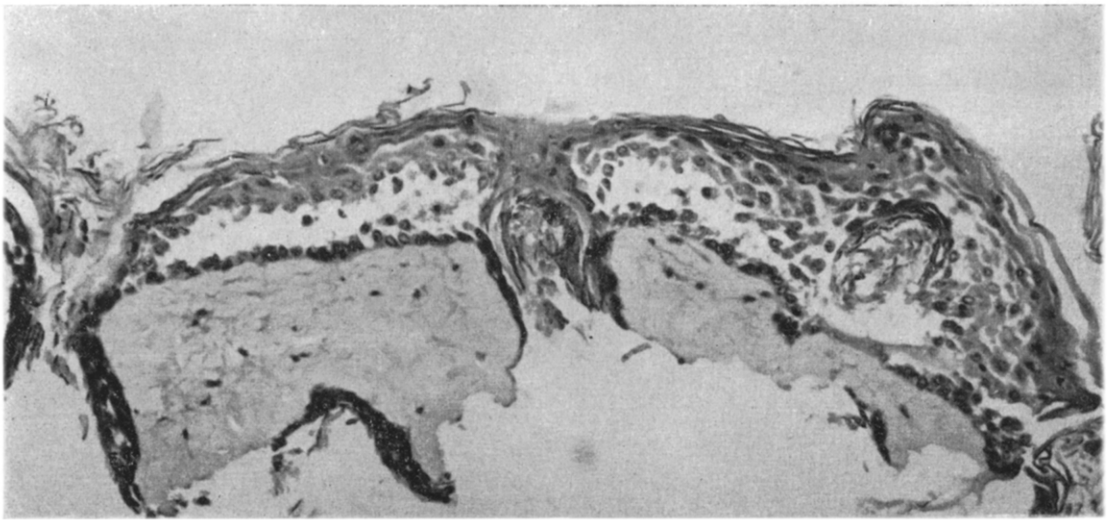

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Fig. 1. (a) *Normal hairless mouse epidermis. The skin has been incubated in trypsin for* 35 *min. To the left is seen the intact, two cell layer thick epidermis. In the right side of the picture the separation has started. The horny layer with the differentiating cells can be lifted off, while the basal cell layer is left on the dermis. By scraping the dermis, the basal cells are obtained in suspension; while the differentiating cells are suspended by shaking the horny layer. The basal cells are afterwards shortly incubated in collagenase to remove contaminating connective tissue fibres.* (b) *The corresponding separation between the basal and differentiating cells in hyperplastic epidermis* 3 *days after application of cantharidin.*

(to face p. 240)

differentiating cells in the same manner also during hyperplasia (Fig. 1).

In one experiment glucose uptake from the medium was estimated by the ortho-toluidine (Hultman) method [13].

For statistical analysis of the difference between painted and unpainted side of the animals a two-sample *t*-test was used. This tested possible significance for differences of the averages of the measured parameters. In the figures only the average ratio painted/unpainted side is shown and not the absolute values of the parameters. Conclusions of differences have only been made when these have been significant. In the tables level of significance is given by + for $p \leqq 0{\cdot}05$ and + + for $p \leqq 0{\cdot}01$.

EXPERIMENTS AND RESULTS

1. *The validity and reliability of the methods*

Normal values for the 4 parameters are given in Table 1. The aerobic lactic acid production is about 44% of the anaerobic one, corresponding to a Pasteur effect of 56%. In some experiments, aerobic lactic acid production and glucose uptake from the medium were measured simultaneously. Average glucose uptake was 18·4 μg per mg dry tissue per hour (SD = ±8·4). An aerobic lactic acid production of 11·3 μg/mg/hr corresponds to a utilization of about the same amount of glucose. This means that 61% of the glucose uptake can be accounted for by the aerobic lactic acid produced. It is therefore assumed that the lactic acid production measured in this way may be taken as an estimate for the rate of aerobic glycolysis. It is also assumed that the same measurements during anaerobic conditions are directly proportional to the rate of anaerobic glycolysis.

Table 1

		SD	No. of mice
Oxygen consumption on endogenous substrate (QO_2) (μl/mg/hr)	3·13	±0·71	27
Oxygen consumption with glucose in medium (QO_2) (μ/mg/hr)	2·83	±0·66	40
Aerobic lactic acid production (μg/mg/hr)	13·3	±3·6	21
Anaerobic lactic acid production (μg/mg/hr)	30·4	±7·1	29

Normal values for oxygen uptake and lactic acid production of skin pieces from hairless mice.

It can be seen that all methods display a rather large standard deviation, reflecting wide variations between the individual mice. This can be eliminated when each mouse serves as its own reference [14]. In all the painting experiments a normal control has been taken from the unpainted side of the mouse, and the results have been expressed as ratio between painted and unpainted side. By this procedure one has avoided that small changes due to the painting should become hidden by large spontaneous variations in the individual groups of mice during an experimental course of several years [14].

It has earlier been shown that isolated basal and differentiating epidermal cells have equal rates of oxygen consumption, and that this rate is well comparable to the QO_2 of intact skin pieces [11].

2. *Painted mice*

The results obtained on skin pieces after a single dose of methylcholanthrene are shown in Fig. 2, and after application of cantharidin in Fig. 3. Each point represents the average of 6 to 12 mice. The corresponding changes in oxygen consumption of suspensions of basal and differentiating cells, as well as isolated dermis are shown in Tables 2 and 3.

The results must be interpreted in the light of known changes in the epidermal cell population kinetics after the same treatment performed in this laboratory [5, 8, 15, 16]. The alterations can be divided into four phases:

(1) An initial *toxic phase* lasting the first 4 hr. With both compounds a uniform depression of the respiration and glycolysis was seen. At the same time mitotic arrest and blocking of the DNA synthesis occur in the epidermis [8, 15,16].

(2) An *intermediary phase* from 4 to 36 hr after the treatment. At this time the proliferative block is released and changes to a more rapid DNA-synthesis and mitotic activity. Many cells are damaged and start falling off. The respiration on endogenous substrate and the lactic acid production showed a temporary increase of moderate degree. This was solely due to the solvent as benzene-treated skin had exactly the same changes. Later the solvent did not provoke any significant changes, and these are therefore not shown separately.

(3) A *growth phase* lasting from 36 to 48 hr after the application characterized by an explo-

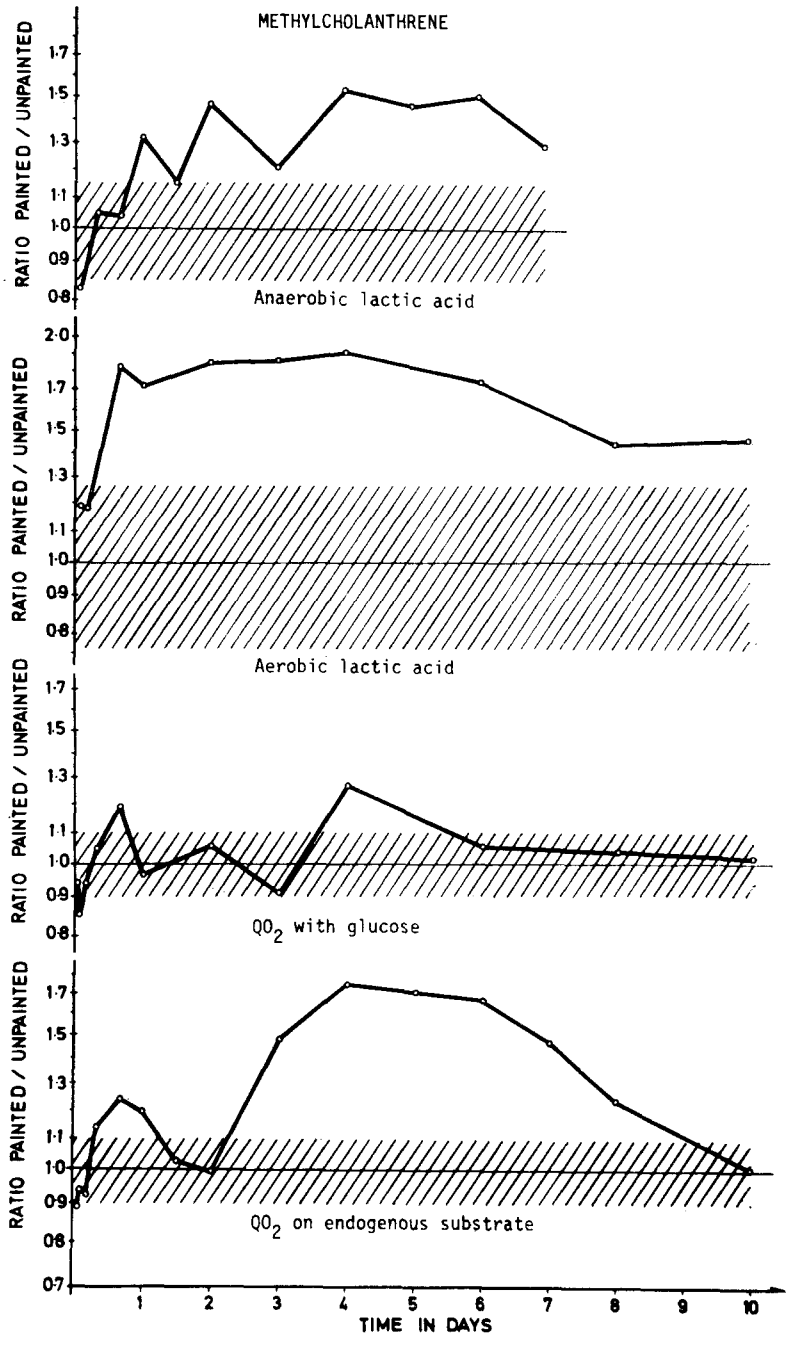

Fig. 2. Oxygen consumption and lactic acid production of skin pieces after a single application of 1% *methylcholanthrene. A rough limit for significance is shown in the hatched areas. (Ranges for* t × *standard error of the mean ratio right/left side of group of* 6 *untreated animals.)*

sive proliferation of the basal cells in MCA-treated as well as cantharidin-treated skin. Most of these cells were then either preparing for division, dividing or recently formed. A pronounced cell loss in the epidermis has now taken place. At the same time the respiration of the carcinogen-treated basal cells was reduced by about 50%, while the glycolysis in the skin was correspondingly increased (Table 2 and Fig. 2). In contrast, cantharidin-treated basal cells with the same degree of proliferative activity had a normal rate of respiration and skin pieces showed only a moderately increased glycolysis (Table 3 and Fig. 3).

(4) A *hyperplastic phase* from 2 to 21 days after the treatment. At this stage the epidermis was 3 to 4 times thicker than normal and consisted mainly of young, immature cells (Fig. 1). The respiration and glycolysis of skin pieces were heavily increased, but not to the same degree as the increase in the number of epidermal cells. When single cell suspensions of MCA-treated skin were measured, their individual respiratory activity was 40 to 50% lower than normal (Table

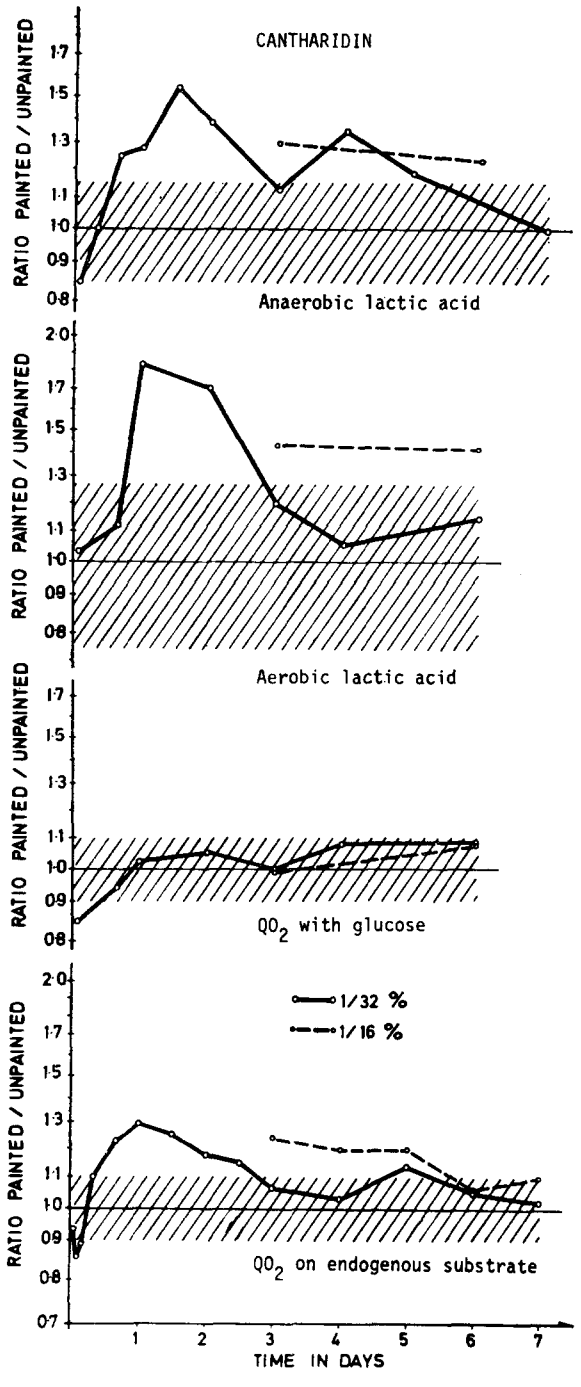

Fig. 3. Oxygen consumption and lactic acid production after a single application of cantharidin to the hairless mouse epidermis.

3). During the hyperplasia the mitotic rate was still increased after MCA and cantharidin treatment [16]. The respiration in the isolated dermis was also increased, but still it was so low that it did not add much to the total rate of oxygen consumption (Tables 2 and 3).

In Figs. 2 and 3 it is seen that the relation between QO_2 with and without glucose, and also between aerobic and anaerobic glycolysis are not constant at the different time intervals

Table 2. Oxygen consumption of basal and differentiating epidermal cells and isolated upper dermis after application of 1% methylcholanthrene and 1/32% cantharidin to the skin. All values are expressed as QO_2 and represent the pooled sample of cells from several mice in each experiment

Days after application	2	2	3	4
(a) *Methylcholanthrene*				
No. of mice	14	10	17	10
Basal cells	3·90	3·94	4·83	1·93
Differentiating cells	3·91	2·59	4·17	2·10
Dermis	0·20	—	0·43	0·80
(b) *Cantharidin*				
No. of mice	8	8		
Basal cells	5·27	4·29		
Differentiating cells	2·25	2·10		
Dermis	0·22	—		

after treatment. This indicates that the Crabtree and Pasteur effects are changing, and this is shown for the different phases in Tables 4 and 5.

Initially, benzene alone provoked a high increase of the *Crabtree effect*, MCA a moderate and cantharidin no increase. The changes were detectable and significant after 2 hr. At maximal proliferative activity after 2 days, MCA treated skin showed practically no Crabtree effect, while cantharidin- and benzene-treated skin had approximately normal values. One day later, when the hyperplasia had been established, the Crabtree effect in MCA-treated skin was nearly 6 times higher than in normal skin. Glucose in "physiological" concentration (1 mg/ml) then inhibited the respiration by 55·4%. The Crabtree effect remained high until the hyperplasia was gradually reversed after 10 days.

During the same degree of hyperplasia induced by 1/16% cantharidin the Crabtree effect was increased to 28·4%, and with half the concentration and less hyperplasia the Crabtree effect was only slightly increased. The increase in Crabtree effect was thus proportional to the hyperplasia. The carcinogen gave the most profound changes. Benzene-treated skin had a transient increase of the Crabtree effect 4 days after application, normalized again on the 8th day.

The Crabtree effect observed in the skin pieces by Warburg technique occurred after a latency time of about 20 min and was maximal about 30 min after the addition of glucose. It was rapidly reversed by 2,4-dinitrophenol to QO_2 values above the initial. The same pattern was also observed in hyperplastic skin where the degree of inhibition and also the reversal by

Table 3. Comparison between average QO_2 values of normal and methylcholanthrene treated epidermal cells and isolated upper dermis. Number of experiments are indicated in brackets

	QO_2 treated cells	QO_2 of untreated cells	Ratio treated untreated	Significance of difference by *t*-test
Basal cells	3·65 (4)	6·54 (5)	0·56	++
Differentiating cells	3·19 (4)	6·56 (7)	0·49	++
Dermis	0·41 (3)	0·16 (3)	2·56	

Table 4. The Crabtree effect of skin pieces at various stages after painting

Time:		2 hr	2 days	3	4	6	8	10
MCA		20·7	3·5	55·4	34·7	43·0	21·6	8·3
Cantharidin	1/32%	10·7	10·8	15·5	5·0	6·4		
	1/16%			28·4		7·8		
Benzene		33·6	6·3	4·2	23·2		7.5	
Normal skin:		9·6 ± 3·8 (s.e.)						
		95% confidence interval 2·0–17·2						

The effect is given in per cent and is calculated by the following formula: (QO_2 on endogenous substrate $-QO_2$ with glucose in the medium)/QO_2 on endogenous substrate. Initially benzene gives the strongest alteration of the Crabtree effect, while during hyperplasia MCA-treated skin has the highest effect. Each single value represents the average difference between two groups of 6 to 12 mice each.

Table 5. The Pasteur effect of skin pieces at three different phases after painting (percentage)

Time	2 hr (toxic phase)	2 days (growth phase)	4 days (hyperpl. phase)
MCA	37	42	47
Cantharidin	47	45	52
Benzene	48	49	56
	Normal Skin 56		

The effects of 1/32% cantharidin are listed after 2 hr and 2 days, while the value after 4 days is representing the maximal hyperplasia due to 1/16% solution. MCA gave the strongest reduction of the Pasteur effect, and these changes are significantly different from the control values with benzene ($p \leqq 0{\cdot}05$). Each value represents the average difference between two groups of 6 to 12 mice each.

dinitrophenol was more pronounced (Fig. 4). This late-occurring respiratory depression by glucose was the only kind of Crabtree effect that could be observed both in normal and in painted skin. During the oxygraphic measurements of suspensions of normal as well as treated basal and differentiating epidermal cells, no immediate effect by glucose addition could be seen during an incubation time up to 10 min.

The *Pasteur effect* showed only relatively small changes at the different intervals after painting with cantharidin as well as with MCA. Changes in the anaerobic glycolysis were therefore roughly followed by similar changes of the aerobic glycolysis. But also here, the most pronounced (and only significant) changes in the Pasteur effect was most reduced initially after the painting, and thereafter only small changes were seen. The Pasteur effect is therefore not listed separately in the table for each day after the different treatments.

DISCUSSION

The toxic phase

Methylcholanthrene and cantharidin cause severe damage to the epidermis. Several structures and functions of the cells are impaired or

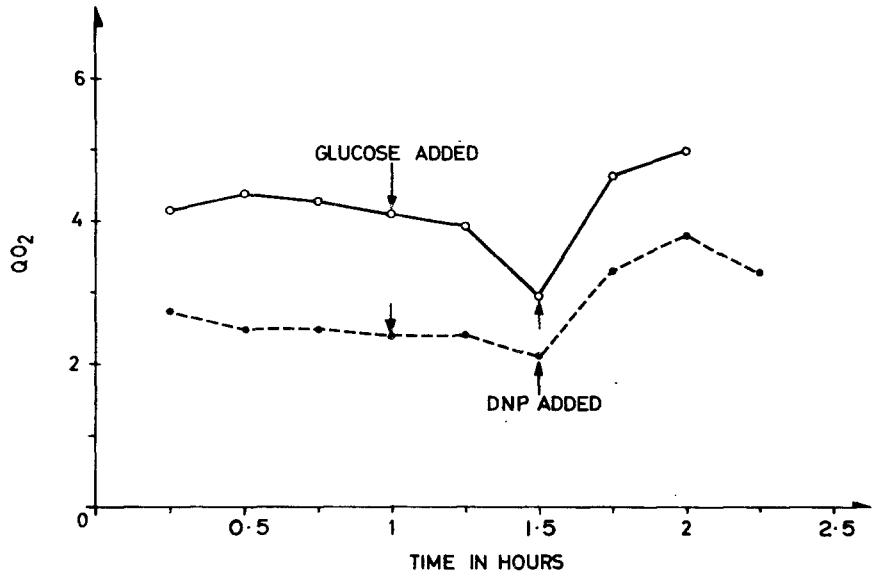

Fig. 4. The Crabtree effect in hyperplastic skin 5 days after one application of methylcholanthrene (above). The corresponding effect in untreated skin is shown in the dotted curve.

destroyed. These damages appear after only a few hours in the so-called "toxic phase". A survey of the alterations of the respiration and glycolysis as well as other parameters studied in the hairless mouse skin is given in Table 6. MCA causes a blocking of the DNA synthesis as well as the mitosis, and the respiration and anaerobic glycolysis are lowered. The latter feature was also seen after cantharidin application, but the mitotic rate was not affected. It may therefore appear that cantharidin has a different mode of action although the resulting cell loss is of the same magnitude for both compounds.

The metabolic changes may be compatible with previous reports that cantharidin causes a blocking of the mitochondrial function [17, 18]. But that is not the sole explanation, as also the glycolysis was affected. Similarly cantharidin inhibits both respiration and glycolysis in liver tissue [17].

A significant feature of the MCA treatment was also that the Crabtree effect was increased while the Pasteur effect was reduced. But this initial change of the Crabtree effect is probably unspecific as the solvent benzene gave a still higher increase of the Crabtree effect. An alteration of the Crabtree effect per se does therefore not appear to be directly linked to any specific immediate effect of the carcinogen.

It is important to note that the initial metabolic depression caused by MCA and cantharidin was of a magnitude of about 20%. This is not much when one takes into account that most of the epidermal cells then are irreversibly damaged leading to a cell loss of about 80% in the following 2 days [8]. By cell loss in this connection is meant the sum of the "physiologic" cell loss due to keratinization and the loss due to induced damage.

It therefore appears that even irreversibly damaged cells are capable of exerting a certain metabolic activity until they are shed a few hours or days later.

The interpretation of the initial effects of the two compounds can be done on a pure toxicological basis because at this interval the histology of the tissue is the same as before the treatment. By this is meant that we study the same cells which were treated, although a severe damage with intracellular changes has taken place.

But when the treated cells after a day or two are shed, a secondary growth with subsequent hyperplasia takes place. The cells are now others than the initially treated ones. Due to the hyperplasia there is more epidermis in relation to connective tissue in the skin. There are more differentiating cells than basal cells. The average age of the differentiating cells is lower than in normal skin, and the basal cells have quite another proliferative rate than an untreated basal cell layer [16]. The epidermal cell population has therefore been profoundly altered and can as such not be directly compared to the same area of skin before treatment.

The growth phase

The changes during the stage of maximal epidermal growth are listed in Table 7. These include the results of own observations as well as other parameters studied by other workers in this laboratory [8, 16, 17]. The results can be

Table 6. *Survey of the relationship between cell population kinetics* [5, 8, 15, 16] *and metabolic parameters in the toxic phase after application of benzene, MCA and cantharidin. The metabolic values refer to skin pieces when not otherwise stated. The tetrazolium method* [15] *refers to pure epidermis*

Parameter	DNA synthesis	Mitotic rate	Mitotic duration	Cell loss	Epidermal cell number	Tetrazolium method	QO_2 endogenous	QO_2 with glucose	Aerobic lactic acid	Anaerobic lactic acid	Crabtree effect	Pasteur effect
Benzene	Increased (Evensen)	Increased (Evensen) Normal (Elgjo)	Normal (slightly lowered) (Elgjo)	Normal (Skjaeggestad)	Normal	Slight decrease (Iversen)	Increased after 1 hr, later normal	Pronounced decrease	Moderate increase	Normal	Increased	Slightly reduced
MCA	Blocked (Evensen)	Blocked 4 hr (Evensen)	Very long (blocking) (Elgjo)	Normal (Skjaeggestad)	Normal	Normal (slightly increasing)	Decreased	Decreased	Slightly increased	Decreased	Normal	Moderately reduced
		Blocked 12 hr (Elgjo)										
Cantharidin		Normal (Elgjo)	Increased (Elgjo)	Normal (Skjaeggestad)	Normal		Decreased	Decreased	Normal	Decreased	Normal	Reduced

Toxic phase 0–24 hr.

Table 7. Survey of the relationship between cell population kinetics and metabolic parameters in the growth phase after application of benzene, MCA and cantharidin. The metabolic parameters refer to skin pieces when not otherwise stated

Parameter	DNA synthesis	Mitotic rate	Mitotic duration	Cell loss	Epidermal cell number	Tetrazolium method	QO_2 endogenous	QO_2 with glucose	Aerobic lactic acid	Anaerobic lactic acid	Crabtree effect	Pasteur effect
Benzene	Normal (Evensen)	Normal (Elgjo, Evensen)	Prolonged (Elgjo)	Normal (Skjaeggestad)	Slightly increased	Normal (Iversen)	Normal Skin pieces: normal	Normal	Moderately increased Decreasing	Normal (slightly increased)	Normal	Slightly reduced
MCA	Increased	Highly increased (by 2½ times) (Elgjo)	Normal (Elgjo) Short (Evensen)	Highly increased (Skjaeggestad)	Moderately increased	Decreasing (Iversen)	Basal cells: low Differentiating cells: low Skin pieces: increased	Normal	Highly increased	Highly increased	Normal	Reduced
Cantharidin		Highly increased (Elgjo)	Short (Elgjo)		Moderately increased		Basal cells: normal Differentiating cells: low	Normal	Highly increased	Highly increased	Increased	Slightly reduced

Growth phase 36 to 48 hr.

directly compared as the same strain of mice and the same painting method was used.

At this point the effects due to the solvent have subsided, and thereafter only very small changes were seen with benzene treatment alone.

Unexpectedly MCA-treated skin pieces at this stage showed "normal" rate of respiration despite the very high proliferative activity, including high DNA synthesis as well as high mitotic rate. However, the total oxygen consumption of skin pieces is the sum of several different factors, and this is revealed when the basal and differentiating cells as well as the dermis are studied separately (Table 2a, b). It is then seen that basal and differentiating cells each have a very low rate of oxygen consumption, but this is outweighed by the increasing number of cells due to the high proliferation. The increase of oxygen consumption of the dermis, which does not add much to the total QO_2, is probably due to a certain inflammatory reaction caused by the painting.

During the growth phase there was an important difference between MCA- and cantharidin-treated skin. The basal cells in the first case had a very low oxygen consumption while in the latter they showed a normal rate (Table 2b). This conclusion is strongly supported by comparing the QO_2 of cell suspensions to the corresponding QO_2 on endogenous substrate of skin pieces after MCA (Fig. 2, bottom curve) as well as cantharidin treatment (Fig. 3, bottom curve). One might speculate whether this feature of carcinogen-treated basal cells could have relation to the finding by Graffi [20], that carcinogens bind to and damage the mitochondria of the cells. A damage due to this binding could then be revealed in the critical phase when these cells are going to divide. The cells therefore have to depend more on the glycolysis. The lack of Crabtree effect could be explained in the same manner. Respiration is already inhibited, the glycolysis is high, and glucose addition does not alter this pattern further. Iversen [15] when measuring the formazan deposits in carcinogen-treated skin, also concluded that a mitochondrial damage had occurred.

Another possibility is that a precancerous metabolic pattern is uncovered during the high proliferation, which must be a very critical phase for cells having been subject to a profound carcinogen damage. The high glycolysis and low respiration in this phase is very similar to the metabolism in tumours discovered in the classical studies by Warburg and his colleagues [21]. In a more recent study, Burk *et al.* [22] similarly found that even the lowest degree of malignancy in Morris hepatomas was accompanied by an increase in the glycolytic capacity. In addition progression in malignant growth was associated with a corresponding increase in fermentation capacity. However, in the present experiments the solvent caused an immediate and rather short increase in the glycolysis. A high glycolysis as such is therefore not necessarily connected with malignancy. Still the direct correlation between high glycolysis, low respiration and explosive proliferation of carcinogen-treated epidermis is suggestive of a specific cellular alteration.

It is known that undifferentiated tissue [23] as well as hepatomas [2] have a lower number of mitochondria than normal cells. Similarly, low respiratory activity of growing MCA-treated basal cells could be explained by a low content of mitochondria. No final conclusion can be drawn, as other carcinogens have not been investigated.

The hyperplastic phase

Epidermis is here composed of many young cells, each of them with a very low respiratory and glycolytic activity. But the number of cells is so high that altogether the total QO_2 and lactic acid production becomes higher than in untreated skin.

When the rate of cell renewal (mitotic rate, DNA-synthesis) gradually returns to normal during this stage, it is during the first days not balanced by a normal cell loss. There are simply no mature cells to be lost. Because of this "synchronizing effect" of methylcholanthrene as well as cantharidin treatment one could expect that many of these newly-formed epidermal cells have not yet started their synthesizing processes. That would explain why they exert such a low metabolic activity, which is far lower than normal keratinizing epidermal cells in the hairless mouse [11].

It is a well-known phenomenon that glycogen accumulates in the epidermis during inflammations as well as during hyperplasia and regeneration. Argyris [24] reported that glycogen was found in increased amounts in the epidermis after treatment with methylcholanthrene. It is possible that this accumulation in some cases occurs because the cells are less active than normally. However, in the hairless mouse epidermis no accumulation of glycogen could be traced by PAS-staining from 3 to 7 days after painting with methylcholanthrene [the author, unpublished results]. It therefore seems that the tendency to glycogen deposits in the epidermis during hyperplasia may vary with different

Table 8. Survey of the relationship between cell population kinetics and metabolic parameters in the hyperplastic phase after application of benzene, MCA and cantharidin. The metabolic parameters refer to skin pieces when not otherwise stated

Parameter	DNA synthesis	Mitotic rate	Mitotic duration	Cell loss	Epidermal cell number	Tetrazolium method	QO_2 endogenous	QO_2 with glucose	Aerobic lactic acid	Anaerobic lactic acid	Crabtree effect	Pasteur effect
Benzene	Normal (Evensen)	Low after 3 days, later normal	Prolonged (Elgjo)	Normal (Skjaeggestad)	Normal	Normal	Normal	Normal	Increased after 4–6 days	Normal, small increase after 4 days	Normal	Normal
							Skin pieces: highly increased					
MCA	Increased (Evensen)	Increased, new peak after 5 days (Elgjo)	Prolonged	Highly increased 3rd day, later decreased (Skjaeggestad)	Highly increased (by 3 times)	Decrease	Basal cells: low Differentiating cells: low	"Normal" moderately increased after 4 days	Highly increased	Highly increased	Highly increased	Slightly reduced
Cantharidin		Increased, slowly decreasing	Prolonged	Highly increased until 4th day (Skjaeggestad)	Highly increased		Moderately increased	"Normal"	Moderately increased	Moderately increased	Increased	Slightly reduced

Hyperplastic phase 2 to 21 days.

strains of mice. The low metabolic activity in hyperplastic epidermis in the hairless mice is as far as the present experiments reveal, not related to any tendency to storage of glycogen.

The low metabolic activity in the carcinogen-treated hyperplastic epidermis could theoretically be due to traces of methylcholanthrene which remain in the skin and exert a continuous influence upon the metabolism of the cells. But as the same pattern of low respiration and glycolysis also was found in hyperplasia induced by cantharidin, it is more likely that this is an unspecific phenomenon. It is also interesting to note that the hyperplasia induced by cantharidin is of the regenerative type with no evidence of any continuous influence of the compound [16].

The Crabtree effect

The inhibiting effect of glucose upon the respiration has been observed in two ways. The first, including Crabtree's original study [1], is by means of manometric techniques. These techniques require an equilibration time of several min before the first reading after the glucose addition. Then in the following 15 to 20 min the depression of the respiration occurs. This could be called the *late* Crabtree effect. Most of the recent studies of this phenomenon, however, has been performed with suspensions of ascites tumour cells, using polarographic techniques. In this way the total incubation time is within the limit of about 10 min, and changes in the rate of oxygen consumption can be read within seconds after addition of a substrate [25, 26]. Such rapid changes would have been overlooked with the manometric techniques. Though it should be pointed out that it is by no means necessary that an initial effect is always present. Exceptions also occur, even in ascites tumour cells [27].

In the present experiments only a late Crabtree effect could be observed with oxygraphic measurements of suspensions of basal and differentiating cells.

The magnitude of the Crabtree effect in normal hairless mouse skin is the same as that found by Gilbert [28] for human skin. He demonstrated a respiratory control mechanism in the human epidermis. As in his experiments, dinitrophenol reverses the effect in hairless mouse epidermis to respiratory values above the initial ones (Fig. 4).

As normal skin exerts a clear Crabtree effect, a study of this phenomenon during carcinogenesis will only be a question of a possible quantitatively-altered effect.

As a high Crabtree effect is considered as typical for tumour tissue, one might also expect that this effect was specifically increased during carcinogen treatment of a normal tissue. According to the present experiments this is apparently not true. First of all, during the initial toxic phase, MCA-treated skin has only a moderately increased effect, while the solvent alone gives a pronounced effect (Table 4). It therefore seems that the initial increase of the Crabtree effect may solely be due to the solvent benzene. Secondly, after 24 hr all the changes in both MCA and cantharidin-treated skin also seem to be due to the solvent. In this case the solvent causes an increase in the respiration which is not seen when glucose is present. This could be called an indirect Crabtree effect.

When the carcinogen provokes a very high proliferation after 48 hr, there is practically no Crabtree effect at all; cantharidin-treated skin with a similar degree of proliferation exerts a normal Crabtree effect. It therefore appears that an increase of the cell proliferation in the epidermis will not increase the Crabtree effect.

However, as a result of the increased growth, a hyperplasia develops, and these new, young cells have a very high Crabtree effect. In this stage the carcinogen-treated skin had the highest Crabtree effect, where physiological doses of glucose would depress the respiration by nearly 60%. This occurred despite the fact that the hyperplastic cells already had a low respiratory rate on endogenous substrate. It also seemed that the magnitude of the Crabtree effect was directly proportional to the degree of hyperplasia after cantharidin treatment, but still the carcinogen gave the most pronounced effect.

Possible mechanisms for the Crabtree effect on the molecular level has been proposed in many investigations [27, 29–33]. But the crucial point in the present experiments is why the effect is lacking during carcinogen-induced growth. Several explanations are possible:

(1) An exhaustion of endogenous substrate due to the high rate of synthesis in preparation for mitosis in the basal cells. In this case adenine nucleotides would be available for the metabolization of glucose, and the respiratory rate after glucose addition would be unaltered or increased. Such a lack of substrate is less likely as the endogenous respiration was constant throughout the whole observation period of 1 hr, and no decline was observed. Endogenous substrate sufficient for maintaining the respiration *in vitro* thus seemed to be present in skin pieces also during the growth phase.

(2) A persistent mitochondrial damage. As a

large amount of pyridine nucleotides would then be available from the destroyed mitochondria, a competition between the glycolytic and the respiratory pathways for coenzymes would not occur. The possiblity of a mitochondrial damage as an important, and maybe specific, effect of carcinogens exists and would be the same as concluded by Iversen and Evensen [15]. The decreased respiratory rate in the basal cells which also remains throughout the hyperplastic phase is very suggestive. This is clearly in contrast to the cantharidin-treated skin where the basal layer seemed to be repopulated with cells with a normal respiratory rate (Table 2).

These features of the Crabtree effect in the skin are in concordance with reports from other tissues. Thus Kiricuta *et al.* [34] found from studies of human tumours that even if the primary tumour exerted a Crabtree effect, this effect was not necessarily present in metastases from the same tumour. Similarly, Laszlo [35] reported that leucemic lymphocytes had a low aerobic glycolysis and no Crabtree effect. The Crabtree effect as such is therefore not necessarily related to malignancy although it is present in most cases. As the present experiments demonstrate, the Crabtree effect neither is necessarily present in rapidly growing cells. Mustea and Muresan [36] reached the same conclusion from metabolic studies of bacterial cultures. However, an increased Crabtree effect, at least in the skin, seems to develop as a *result* of increased proliferation during hyperplasia. It therefore seems that the respiratory and glycolytic patterns as well as the Crabtree effect are entirely different in actually proliferating and in newly-formed, hyperplastic cells.

The Pasteur effect

From Table 5 it is seen that a reduced Pasteur effect in the skin can be observed in direct toxic damage of the cells as well as during growth and hyperplasia after MCA treatment. During the toxic phase as well as the hyperplastic phase this means that the relative decrease of the anaerobic glycolysis *per cell* is more pronounced than the corresponding aerobic glycolysis.

However, in the growth phase the Pasteur effect is reduced at a very high rate of anaerobic glycolysis, meaning that the aerobic glycolysis is even more increased. From a teleological point of view this reduction of the Pasteur effect might be explained by an urgent need for glycolytic energy during high proliferation. But, as the reduction of the effect here is rather small, it would not account for much increase in energy released.

The Pasteur effect has been explained to occur partly due to a competition between glycolysis and respiration for ADP [37, 38] and partly as a competition for inorganic phosphate [39, 40]. Such a competition would be a likely explanation during the growth phase, where the respiration is low and the glycolysis high. However, this can not explain the whole picture due to the lack of Crabtree effect, as discussed earlier.

In any way, the experiments demonstrate that in hairless mouse skin the Pasteur effect can be moderately diminished both at high and low glycolytic rates, and these changes are not parallel to the Crabtree effect.

The main conclusion of these investigations is that most of the effects of a single application of a strong carcinogen as MCA upon epidermal respiration and glycolysis are unspecific when compared to similar treatment with irritants. The changes are mostly due to altered cell population kinetics in epidermis as a whole, but they also reflect changes of the metabolic rate within each single cell. The only possible specific feature of MCA treatment was the very high glycolysis and low respiration of maximally proliferating cells. As cellular proliferation is critical for later cancer development, this pattern may reflect changes which are directly related to carcinogenesis.

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Antitumour and Haematological Effects of δ-oxygen Substituted Butyl Ethers

M. J. TISDALE, L. A. ELSON and W. C. J. ROSS

Chester Beatty Research Institute, Institute of Cancer Research Royal Cancer Hospital, London, S.W.3, England

Abstract—*The haematological response produced by some representative δ-oxygen substituted butyl ethers closely resembled that shown by aromatic 2-chloroethylamines implying a similar mechanism of biological action. Kinetic evidence from the rate of hydrolysis of such compounds would also lend support to this idea. The compounds were effective inhibitors of the Walker rat carcinoma.*

ALTHOUGH β-nitrogen and β-sulphur atoms considerably enhance the hydrolytic rate of alkyl halides over the parent unsubstituted compound, the same is not true of β-oxygen atoms which cause a large retardation in rate [1]. Both nitrogen and sulphur form stable five-membered ammonium and sulphonium salts, but oxygen does not favour stable oxonium salts. Thus although there is a great tendency to form a five-membered ring, the ring is not stable. Alkylation of external nucleophiles is therefore possible, and δ-oxygen substituted alkyl halides should be able to alkylate centres in biological molecules. The proposed mechanism of action of these compounds bears a close relationship to 2-chloroethylamines and 2-chloroethylsulphides and it was anticipated that compounds of this type would display a similar effect on the haemopoietic system. It has been shown [2] that halogenated butyl ethers have a similar mechanism of mutagenic activity.

METHODS

Bis-(4-bromobutyl)ether, prepared by the method of Sieber [3] gave an oil, b.p. 93–96°C/0·5 mm ([3], b.p. 98–104°/0·6 mm).

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Bis-(4-methanesulphonoxybutyl)ether, gave a semi-solid from ethanol, m.p. 14–15°C ([3] m.p. 15·5°C).

Acetaldehyde-bis(4-chlorobutyl)acetal had b.p. 96–98°C/12 mm ([4] b.p. 109–110°/30 mm).

Determination of hydrolysis rates

Hydrolysis rates were determined using a "Radiometer titrator" (TTTI).

The compound (0·02 m-mole) in acetone (0·1 ml) was introduced into a thermostatted vessel at 60°C, containing 0·1 M potassium perchlorate solution (30 ml) at pH 7. The solution was automatically maintained at this pH by the continuous addition of 0·1 N sodium hydroxide from a micrometer burette, and the rate of addition was recorded on a chart using the "radiometer titrigraph" (S.B.R.2).

Anti-neoplastic assay

The protocol for testing compounds for inhibitory action against the Walker rat carcinoma is described by Connors *et al.* [5]. Compounds were administered in 10% acetone in arachis oil by a single intraperitoneal injection on the day following implantation. In Table 1 the C/T ratio gives the weight of the control tumour/the weight of the treated

Table 1. Anti-tumour assays against the Walker rat carcinoma and hydrolysis rates (kH_2O) *at pH 7 and 60°*

Compound	$k_{H_2O} \times 10^3$ min^{-1}	Dose mg/kg	C/T ratio	LD_{50} (mg/kg)	ED_{90} (mg/kg)	T. I.
$O(CH_2CH_2CH_2CH_2Br)_2$	40·5	75	3·5	395	92	4
		100	∞			
		250	∞			
$O(CH_2CH_2CH_2CH_2OSO_2CH_3)_2$	333	0·2	3·0	2	0·3	6
		0·4	∞			
		0·8	∞			
$Cl(CH_2)_4OCHO(CH_2)_4Cl$ (CH$_3$ on CHO)	3·3	240	1·1	>1500	1240	>1
		600	2·0			
		1500	∞			

tumour; LD_{50} is the dose required to kill 50% of the animals; ED_{90} is the dose which will produce 90% inhibition of tumour growth; and the therapeutic index (T.I.) is LD_{50}/ED_{90}.

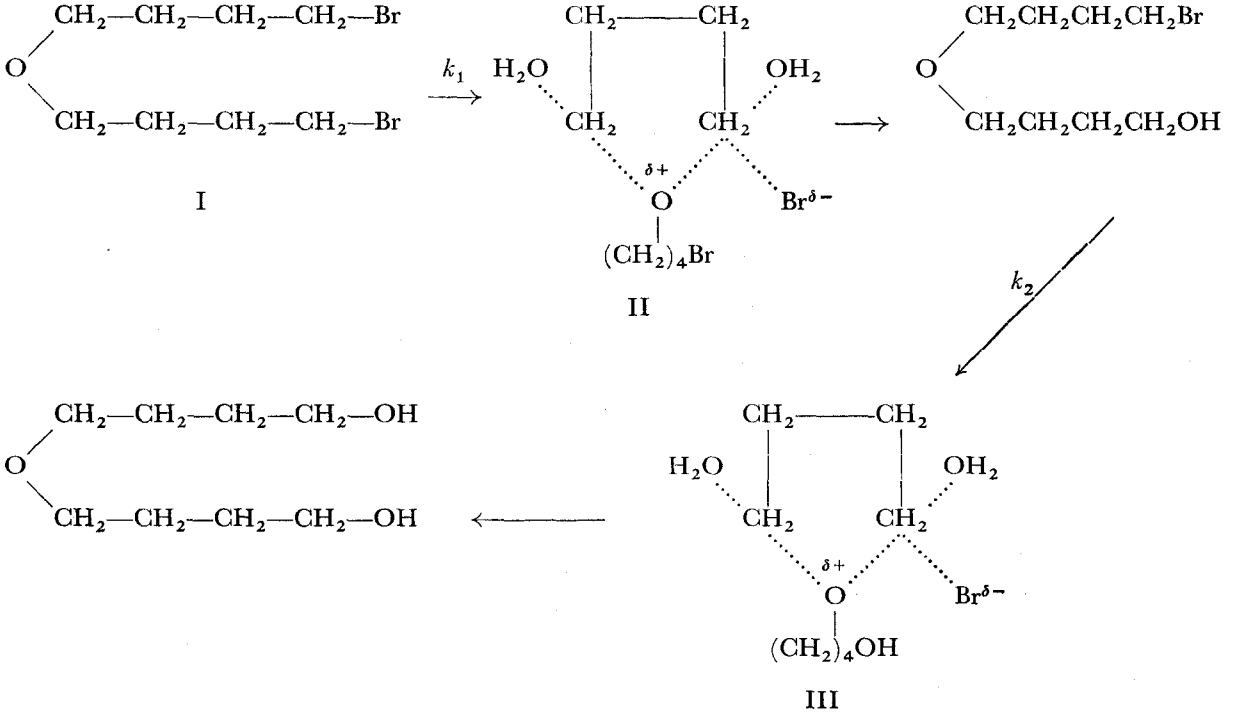
Haematological effects

The procedure for determining haematological effects of drugs is described by Elson *et al.* [6].

RESULTS AND DISCUSSION

The results of screening the compounds against the Walker rat carcinoma and the hydrolysis rates at 60°C are given in the table. A certain level of chemical reactivity is necessary for biological activity as was the case for the arylhalogenoalkylamines [7]. Bis-(4-methanesulphonoxybutyl)ether, although hydrolysing eight times more rapidly than the corresponding bromo-analogue is not a more efficient tumour growth inhibitor because of its high toxicity. These compounds were shown [8] to have appreciable activity against the Ehrlich ascites carcinoma.

In view of the evidence of Blandamer *et al.* [1] for the hydrolysis of 4-bromobutyl-4-methyl ether, it seems reasonable to represent the transition states for hydrolysis of bis-(4-bromobutyl)ether (I) as II and III, i.e. sym-



metrical transition states. Application of the Swain equation [9] to the hydrolysis of I shows that k_1 and k_2, the velocity constants for the hydrolysis of the two arms of bis-(4-bromobutyl)ether, are equal [$k_1 = 7{\cdot}93 \times 10^{-2}\ \text{min}^{-1}$; $k_2 = 7{\cdot}98 \times 10^{-2}\ \text{min}^{-1}$]. This might be anticipated because substitution of hydroxyl for bromine in III would not be expected to have any effect on the lone pairs of electrons on the oxygen atom. The hydrolysis is represented as shown with two simultaneous processes as it seems unlikely that the oxygen electron pairs could be involved in the formation of two tetrahydrofuran rings at the same time.

Institoris *et al.* [10] attributed lymphotoxicity to compounds containing a basic nitrogen atom (2-chloroethylamines and aziridines) and to compounds capable of liberating alkali whilst alkylating (epoxides). Myelotoxicity was thought to be produced by the α, ω sulphonoxyalkanes which contain no basic group, but which are capable of liberating an acid on hydrolysis. They thought it likely that the lack of basicity enables the myelotropic agents to exert a selective inhibitory effect on myelopoiesis without attacking the lymphatic system.

However, an inspection of the series of alkylating compounds producing lymphoid effects will show that in all cases participation by a basic heteroatom is concerned, and the formation of the initial carbonium ion always goes through a stained ring system. In the case of oxiranes and aziridines the compounds exist in unstable three-membered rings in the free state, and factors which tend to weaken this ring will increase their reactivity.

The relative haematological responses for bis-(4-bromobutyl)ether and bis-(4-methanesulphonoxybutyl)ether are shown in Figs. 1 and 2 respectively. With the former compound there is a considerable loss in body weight over the first 5 days. This probably results in haemoconcentration which would account for the initial rise in platelets. The fall in blood neutrophils and lymphocytes would probably be greater if it were not for this haemoconcentration, and bone marrow damage is almost certainly more severe than would be expected from the observed fall in leucocytes. Bis-(4-methanesulphonoxybutyl)ether is a much more potent depressor of lymphocytes, neutrophils and platelets than is the bromo-analogue. In both cases there is a rapid neutrophilia following the initial depression of lymphocytes and neutrophils, and the overall response compares favourably with that obtained with a typical arylhalogenoalkylamine [11].

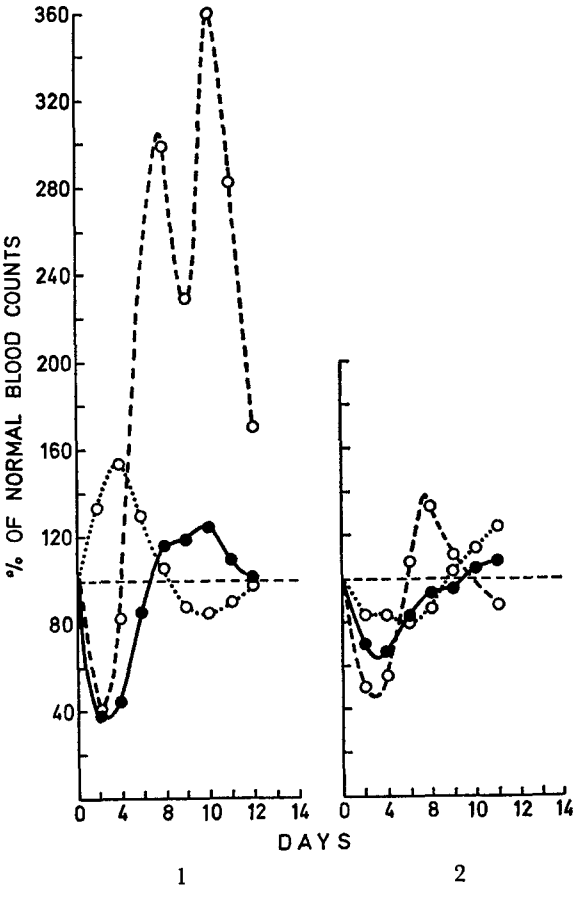


Fig. 1. *Haematological response of* $O(CH_2CH_2CH_2CH_2Br)_2$, 300 *mg/kg. given i.p. in arachis oil.*

Fig. 2. *Haematological response of* $O(CH_2CH_2CH_2CH_2OSO_2CH_3)_2$, 2 *mg/kg given i.p. in ethanol and arachis oil.*

Key:——lymphocytes; · · · · neutrophils; – – – platelets

The haematological responses for these compounds confirm the theory that the determining effect will be the initial cyclisation. Intracellular hydrolysis of bis-(4-methanesulphonoxybutyl)ether would liberate methanesulphonic acid in the same way as would busulphan, but the effect of these two compounds on the haematopoietic system is so different that this cannot be important.

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The Relationship of Somatic Cell Hybridization to Carcinogenesis

RICHARD J. ELKORT, ALFRED H. HANDLER, SIDNEY KIBRICK and LAWRENCE KLEINMAN

Boston University School of Medicine

Abstract—*A cell line derived from normal hamster embryo cells was treated with inactivated Sendai virus, and inoculated into the cheek pouches of Syrian hamsters. Progressive tumor growth resulted at the sites of inoculation. The malignant nature of of these growths was confirmed by successfully allografting tumor fragments to a second hamster, with resultant invasive growth.*

THE RELATIONSHIP OF SOMATIC CELL HYBRIDIZATION TO CARCINOGENESIS

In 1957, Okada [1] showed that certain viruses can induce somatic cells to fuse their nuclei and cytoplasm, forming so-called synkaryons or heterokaryons. Harris investigated this phenomenon in great detail, confirming and extending Okada's initial observations [2, 3]. Cells that have undergone fusion generally remain alive and reproduce, although some alterations are observed in their morphologic characteristics, e.g. varying nuclear/cytoplasmic relationships.

We propose the theory that, under appropriate conditions, cell fusion, spontaneous or virus mediated, results in the appearance of a population of altered cells having the characteristics which we recognize as neoplasia. The following preliminary studies were performed in order to test the validity of this theory.

The 68th passage of a clone taken from the 99th passage of a line of normal hamster embryo cells developed in Dr. John Ender's laboratory at the Children's Hospital Medical Center of Harvard Medical School, (Iota Line), was treated with beta-propriolactone inactivated (killed) Sendai virus. Cell fusion was determined qualitatively *in vitro* by direct observation for 2–4 hr. Twelve 6-week old Syrian hamsters were anesthetized with intra-peritoneal injections of sodium pentobarbital. The fused cell mixture, suspended in phosphate-buffered saline, was inoculated into both cheek pouches of 9 of the hamsters, after the method of Handler and Foley [4, 5]. The injections were made in 0·1 ml volumes in a series of 10-fold dilutions ranging from 10^3 to 10^6 viable cells/0·1 ml. Three animals served as experimental controls and were inoculated with cells that were treated identically with those in the experimental group, except that these control cells had not been exposed to the killed Sendai virus.

Hamsters were maintained on an *ad libitum* diet of standard hamster pellets and water. Cheek pouches were observed at weekly intervals until the death of the animal. Three of the nine experimental group animals and two of the three control group animals were sacrificed 6 to 8 weeks following inoculation so that autopsies could be performed and tissues obtained for histologic examination. These animals were included in the overall results summarized below. One of the experimental group animals died of anesthetic overdose, 5 days after inoculation, and was eliminated from further tabulation.

Nodules appeared within 10 days in 11 of 16 inoculated cheek pouches in the remaining 8 animals of the experimental group. Every animal in that group developed a nodule in at least one cheek pouch, 3 animals had nodules

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in both pouches, while the remaining 5 each had a nodule in one pouch, although their opposite pouch was negative. A single nodule appeared in 1 of 6 inoculated cheek pouches in the 3 control group animals. In the experimental group, nodules enlarged progressively, eventually filling the cheek pouch in several cases. The growths varied in size from 2 to 7 cm in its greatest diameter. The one nodule in the control group animal attained a maximum size of 2 cm in its greatest diameter.

Experimental group animals survived 12 to 22 weeks from the time of inoculation, and there were no survivors in the experimental group, after 22 weeks. The single control animal with a cheek pouch tumor, was sacrificed at 20 weeks.

Complete autopsy of all animals showed no gross evidence of tumor growth except at the site of inoculation in the "positive" cheek pouches. The "negative" pouches were completely normal grossly and microscopically in both the experimental and control groups, suggesting that tumor growth was an all or none phenomenon. Death was probably caused by pressure necrosis of the cheek pouch membrane by the progressively growing tumor, with secondary bacteremia and systemic sepsis. Gross examination of the tumor nodules showed unilocular cystic masses with extensive central necrosis. Histologic study of the solid rim of these tumors revealed a sarcomatous-like pattern with much cellular pleomorphism and many mitotic figures (Fig. 1). The microscopic appearance of the single tumor in the control group was similar to that of the 11 in the experimental group. Representative samples of liver, lung, spleen and bone marrow were taken from all experimental and control animals for histologic study, using hematoxylin and eosin, or Wright's stain. All of these tissues appeared to be normal microscopically, except that several of the spleen sections in the experimental group showed some hyperactivity in the form of lymphocyte proliferation.

In order to confirm the malignant nature of the cheek pouch tumors, two of the growths from the experimental group were allografted subcutaneously into two 10-week old Syrian hamsters. The single growth appearing in the control group animals was similarly allografted into a single hamster. Using sterile technique, the tumor nodules were minced, homogenized in a tissue grinder and suspended in normal saline. Approximately $1{\cdot}5 \times 10^6$ viable cells were then inoculated with a 14 gauge trocar into each of 4 appropriately prepared dorsal subcutaneous sites. Within 21 days, both the animals receiving cells from the tumors in the experimental group had died. Autopsy revealed extensive growth in the dorsal subcutaneous space with widespread invasion of the retroperitoneal space and encapsulation of both kidneys and ureters. There was no clear evidence of metastatic disease and no extension into the renal parenchyma. Death probably resulted from bilateral ureteral obstruction. Histologic examination of representative areas of the tumor mass, showed the same sarcomatous pattern that was observed in the original cheek pouch tumors (Fig. 2). The single hamster bearing allografts of the tumor taken from the control animal was sacrificed at 3 weeks. No evidence of tumor growth was noted in either of the injection sites, and complete autopsy, showed no abnormalities.

Although it has been known for some time that somatic cell hybridization is not a lethal phenomenon [6–7], the long term fate of fused cells *in vivo* has not, to our knowledge, previously been studied except in terms of the giant, multinucleate cells associated with certain granulomatous or viral diseases [8, 9]. It is possible that, under proper conditions, essentially normal cells undergo fusion, *in vivo* and thereby acquire the heritable growth characteristics of malignancy. This response may be dependent upon a variety of factors including the type and number of fused cells, the nature, viability and amount of mediating virus, and key host factors relating to the immune system.

The present initial experimentation tends to support this concept despite the fact that we used a cell line cloned and passaged many times. Transplantability and subsequent invasive growth was observed only in those tumors resulting from the implantation of virus-treated cells. Hamster cells derived from primary cell culture and from fresh normal adult and embryo tissues are currently being examined in this experimental system.

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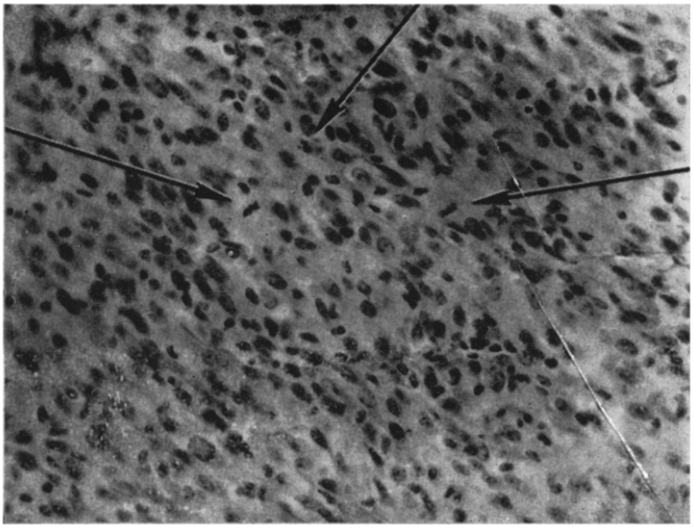

Fig. 1. *Section from a primary tumor resulting from the implantation of virus-treated cells in the hamster cheek pouch. Hematoxylin and eosin;* × 320. *Arrows indicate mitotic figures.*

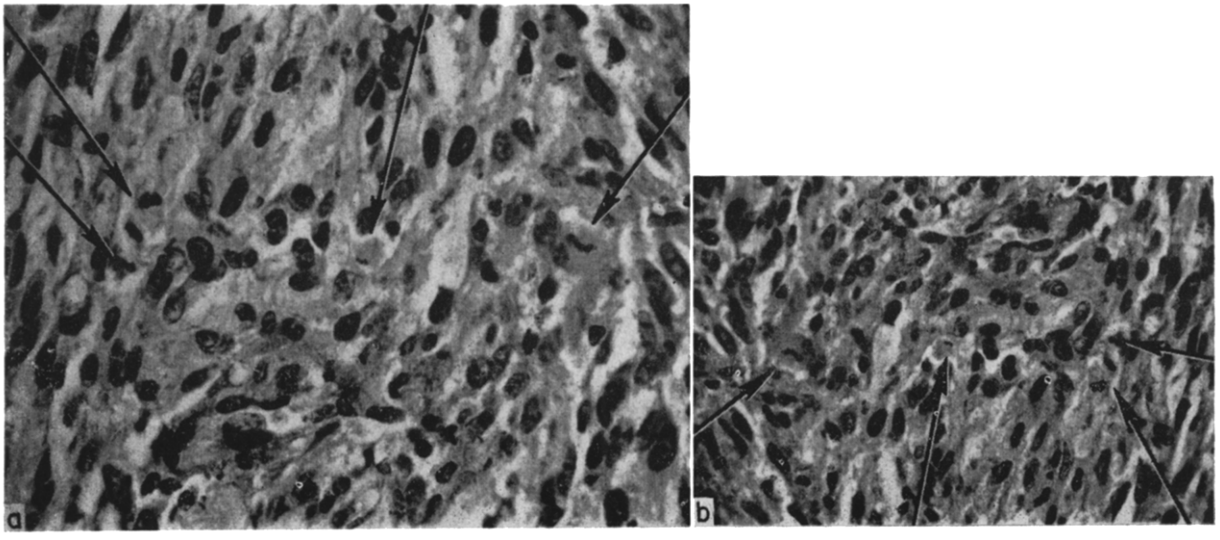

Fig. 2. *Section of a tumor resulting from the allografting of a primary cheek pouch tumor into the subcutaneous space of a second hamster. Hematoxylin and eosin;* (a) × 320, (b) × 480. *Arrows indicate mitotic figures.*

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A Strategy of Chemotherapeutic Eradication based upon Correlative Variations in Total Cell Population, Growth Fraction and Resistance

M. DUCHATELLIER* and L. ISRAEL†

*Laboratoire de Physique des Structures et Systèmes Biologiques (Professeur Barraud), 12, rue Cuvier, Paris V^e, France

†Consultation de Médecine et Groupement d'Etudes et Recherches Cancérologiques, Centre Hospitalier Universitaire Lariboisière, 2, rue Ambroise-Paré, Paris X^e, France

Abstract—*Having developed in previous papers the mathematical connections between growth fraction and cellular losses, and between growth fraction, doubling time, initial cell population, resistance to a given therapy and the number of courses of this therapy per doubling time, the authors examine here how these parameters are connected when eradication is to be achieved. The various calculations show that resistance must be lowered by means of combinations, well beyond the theoretical limits, in order to deal with non-dividing cells and/or large initial tumor cell populations. For a given percentage of resistance, eradication is achieved only for growth fraction above a certain limit, only for tumor cell population below a certain limit, and only for a number of courses of therapy per doubling time above a certain limit.*

In order to meet these requirements, it appears necessary (1) *to combine the available drugs without cross-resistance;* (2) *to give simultaneous combinations instead of sequential ones;* (3) *to treat as soon as possible;* (4) *to find out ways of lowering toxicity in order to give as many courses as possible per doubling time;* (5) *to incorporate in combinations drugs effective against non-dividing cells.*

IT HAS been shown in previous papers [1, 2] that, within the period of constant growth rate of human tumors, that is, grossly between the 30th and the 39th doublings, some parameters are linked with one another by mathematical relations.

(*a*) The growth fraction c and the cellular losses p are linked by the following equation:

$$c(1-p) = \frac{Ln2}{N} \quad [Ln\ 2 = nep.\log.2], \qquad (1)$$

where N stands for the number of cell cycles per doubling time;

(*b*) The growth fraction c, the percentage of initial resistance against the drug or drugs employed r (these drugs being effective only against dividing cells), the doubling time DT, the time elapsed between two courses of the therapy (ti), the number of cells before the treatment is started n_0, and the number of living cells remaining inside the tumor after each course n, are linked by the following equation:

$$n = n_0[r+(1-r).(1-c)^x].2^{(x-1)\,ti/DT}, \qquad (2)$$

where x stands for the number of courses of therapy and where the term $2^{ti/DT}$ implies, by the duration of DT, the existence of cellular losses.

It can be seen that a possibility is thus offered to examine the reciprocal changes of the parameters n_0, c, r, x and $a = ti/DT$ when $n<1$, that is, to consider which are, with respect

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to these reciprocal changes, the conditions for eradication.

We shall consider in the following the connections between c and r, c and a, ; c, r and n_0 ; r, n_0 and x for $n<1$ which represents a good probability of achieving eradication. Then will be discussed the strategic rules which may be deduced from the results thus obtained.

(1) *Correlative changes of growth fraction* (c) *and resistance* (r)

It follows from equation (2) that for any value of n_0, the correlation between c and r can

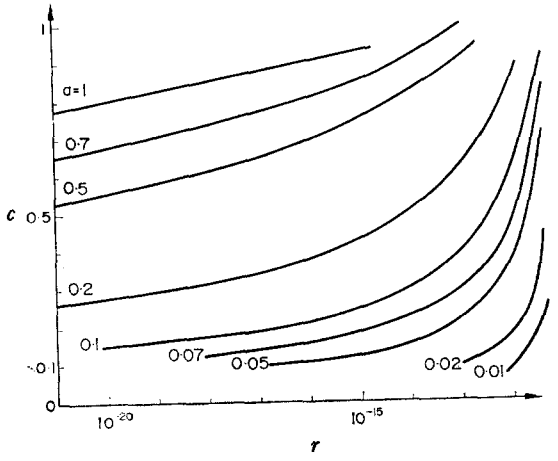

Fig. 1. Correlation between growth fraction c *and resistance* r *for a global population* $n_0 = 10^{11}$ *cells and for* 9 *different values of* a (a = ti/DT ≒ *number of chemotherapy courses per doubling time), if eradication is to be achieved.*

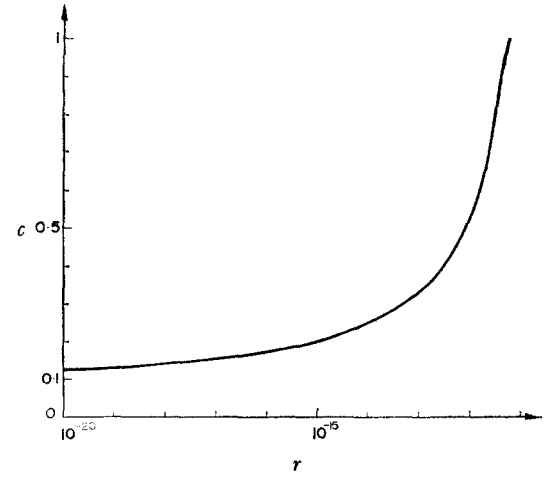

Fig. 2. Development of one of the curves of Fig. 1 : a = 0·07, *with* $n_0 = 10^{11}$. *With* $r = 10^{-12}$, *eradication can be achieved only if* c ≧ 0·5.

be calculated for consecutive values of a. An example with $n_0 = 10^{11}$ and $r = 10^{-12}$ (all cells sensitive) is shown in Fig. 1. It can be seen that for any given value for $r \leqslant 10^{-12}$, the higher the growth fraction, the easier is the eradication, and that, for any given value of c, eradication can be reached only for some values of r, many times lower than 10^{-12}. As an example, Fig. 2 shows $c = f(r)$ for $n_0 = 10^{11}$ and $a = 0{\cdot}07$ (a weekly course for a 100 days' doubling time). If the initial resistance against the therapy employed is $r = 10^{-12}$, eradication is reached for $c \geqslant 0{\cdot}53$. If $c = 0{\cdot}12$, this would require $r \leqslant 10^{-20}$.

(2) *Correlative changes between growth fraction* c *and the ratio of time elapsed between two courses over doubling time* (a)

Figure 3 shows the minimum value of c which allows eradication to be reached, in connection with changes in a, for $n_0 = 10^{11}$ and $r = 10^{-12}$. It appears on the curves that for any given value for c, eradication is reached for some values of a smaller than a certain limit. For example, if $a = 0{\cdot}25$, that is if toxicity allows only 4 courses per doubling time to be given, eradication can be reached only if $c \geqslant 90\%$.

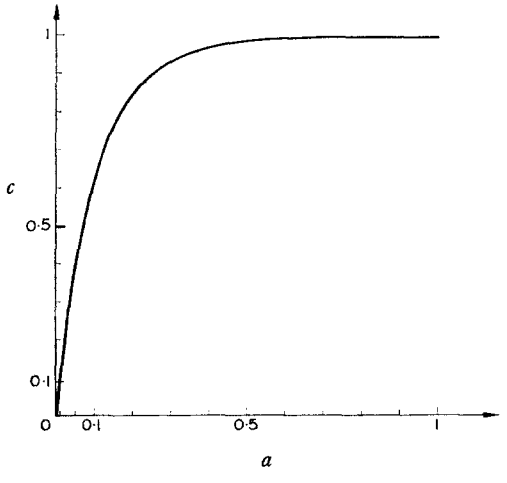

Fig. 3. Correlation between c *and* a, *for* $n_0 = 10^{11}$ *and* $r = 10^{-12}$ *if eradication is to be achieved. The lower* c, *the more courses of chemotherapy must take place within a doubling time.*

(3) *Correlative changes between growth fraction* c, *resistance* r *and initial population* n_0

When choosing an arbitrary value for a, ($a = 0{\cdot}07$ in Fig. 4) it becomes possible to study how c and r correlate for different values of n_0. If we take as an example $c = 0{\cdot}25$, it can be seen that eradicating 10^{11} cells requires $r \leqslant 10^{-14}$ instead of $r \leqslant 10^{-9}$ for 10^7 cells, and $r \leqslant 10^{-4}$ for 10^3 cells. In other words, the product r, n_0 does not remain the same for all tumor sizes and the eradication may be reached with less effective drugs or combinations when the initial population is small.

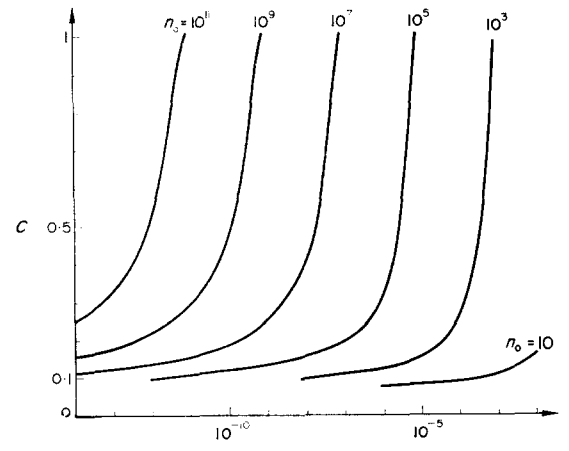

Fig. 4. Correlation between c *and* r *for* a = 0·07 *(one course every 7 days for a* D.T. *of* 100 *days) and for different values of* n_0. *The requirements for* r *decrease faster than* n_0, *so that it is much easier to eradicate small populations, as far as cell sensitivity is concerned.*

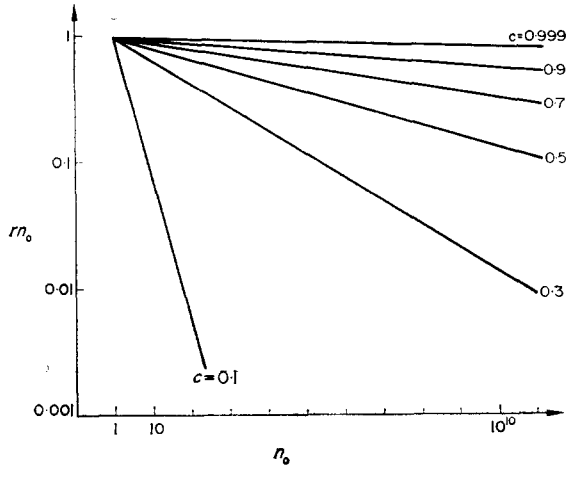

Fig. 5. Correlation between n_0 *and the product* $r.n_0$ *for* 6 *different values of* c.

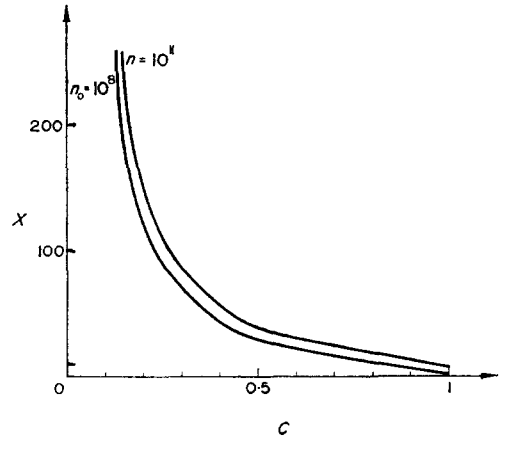

Fig. 6. Connection between c *and* x_0 *(number of courses required for eradication) for* 2 *different values of* n_0. x_0 *depends much more upon* c *than upon* n_0.

(4) *Correlative changes between resistance, initial population and the number of courses required for eradication* x_0

Figure 5 shows, on a logarithmic scale the curves $r.n_0 = f(n_0)$ for different values of c and for $n = 0$ (eradication). It is then possible to construct Fig. 6 which shows the connection between x_0 and n_0 as a function of c. It appears that, for r small enough, x_0 is much more influenced by c than by n_0. As an example, if $c = 0{\cdot}5$, when n_0 varies from 10^6–10^{11}, x_0 varies only from 31–56.

DISCUSSION

I. It must be stated at first that the definition of the percentage of resistant cells r raises some logical difficulties. If $r = 10^{-12}$ with a given drug in a given tumor, that does not mean of course that there is, in a population of 10^{10} cells, one hundredth of a resistant cell, nor that chances are 1% of finding a resistant cell, but only that if the initial population keeps growing, there will be one resistant cell when the total population is 10^{12}, whichever the mechanism is of appearance of this resistant cell.

II. It must be also emphasized that the equation (2), studied elsewhere [2] is valid only if the following requirements are met.

(*a*) The doubling time must be constant between the consecutive courses of therapy, at least during this therapeutic period. This has been shown to be the case by Breur [3], Bru [4] and by us [5, 6].

(*b*) The growth fraction must remain constant during the same period. To our knowledge, this remains to be shown, but there are two reasons for accepting this possibility: (1) it would otherwise prove impossible to predict the tumor growth, and (2) the tumor growth is nevertheless predictable on the basis of a constant doubling time for some doublings, and there is no reason to assume that changing rates of cellular losses adapt themselves to changing values for growth fraction, just in order to keep the doubling time constant, during the period under consideration.

(*c*) The last condition for the validity of equation (2) is that the drugs under study do not interfere with non-proliferating cells. Such is the case in many clinical situations.

III. If it can be assumed that the above conditions are met, the developments of equation (2) lead to some important results with respect to the achievement of eradication.

(*a*) The value of the growth fraction appears to be of the greatest importance, since it can be

seen that below some limits eradication cannot be achieved, no matter how effective the treatment is.

(*b*) But, surprisingly enough, it appears that increasing the effectiveness of the drugs employed, in terms of cell sensitivity, well above the theoretical requirements based upon the total population to be treated and the specific sensitivity of the tumor to the drugs, is of some value in order to deal with the non-proliferating cells. For example, when it is not possible to achieve eradication with $r = 10^{-12}$ and $n_0 = 10^{11}$, according to a given value for c, it becomes possible to achieve eradication with a kind of therapy characterized by $r = 10^{-15}$.

(*c*) But it has been shown also that the higher the parameter r, due to the poor effectiveness of drugs now available, the smaller n_0 has to be, if eradication is to be achieved. For a growth fraction of 25% and a percentage of resistance of 10^{-9} (an extremely good achievement with available combinations) eradication could be reached only for $n_0 = 10^7$, that is a solid tumor of about 2 mm in diameter. In other words, for a given initial population, the lower c is, the lower r has to be; for a given growth fraction, the higher the initial population, the lower r must be. So it is always of benefit, in clinical situations, to meet the lowest possible percentage of resistance, a condition that requires combinations of as many drugs as possible without cross-resistance (considering of course the limitations of toxicity).

(*d*) And of course the lower n_0, the easier it is to achieve eradication. This implies not only that the chemotherapy must be given as soon as possible, but also that the combinations employed must be simultaneous and not sequential, in order not to let the resistant population to drug A keep steadily growing during the administration of drug A.

(*e*) It has also been shown that, given the optimal relations between c, r and n_0, it remains necessary to reach a sufficient number of courses of therapy per doubling time. With respect to this condition some tumors with very short doubling times could prove difficult to eradicate, even with very large growth fractions.

(*f*) As for the total number of courses required in order to reach eradication it depends largely upon the growth fraction, so that it could take longer to eradicate a small tumor with a long doubling time and a small growth fraction than a large tumor with a large growth fraction.

To summarize all the above conclusions it can be seen that the strategy of eradication must take into account the growth fraction, the effectiveness of the therapy with respect to the percentage of resistant cells, the growth rate, expressed as the doubling time, and the number of cells at the beginning of treatment. This strategy may influence the number of drugs to be employed in a combination, the delay between two consecutive courses and the total duration of treatment.

This implies not only a decrease in toxicity for cell cycle sensitive drugs but also for cell cycle insensitive drugs, because it follows, from all the data above, that drugs effective against nondividing cells are a major requirement, as far as chemotherapeutic eradication is concerned, except perhaps for tumors with very short doubling times and very large growth fraction.

Acknowledgement—We want to acknowledge Pr. Baudoin who kindly made available to us the computers in his laboratory. (Requests for reprints should be addressed to L. Israel.)

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Letter to the Editor

Indication of Hypoxic Areas in Tumours from *in vivo* NADH Fluorescence*

MARIO GOSALVEZ†, RONALD G. THURMAN, BRITTON CHANCE and H. S. REINHOLD‡

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104, *U.S.A. and Department of Radiology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.*

THE PRESENCE of hypoxic cells in tumours has been demonstrated by different methods [1–4, 7]. The relations between intracellular anoxia and radiobiological anoxia has been questioned both from the theoretical [5] and from the experimental [6] standpoints. It seems of interest to identify hypoxic areas in tumours by direct measurements of the intracellular redox state. The fluorescence of NADH has been shown to be a sensitive and relatively reliable parameter for the measurement of intracellular hypoxia and the study of metabolic control phenomena [8–12].

A biological preparation uniquely suited for studies of localized hypoxia is provided by a tumour growing in a sheet-like fashion (so called "sandwich" tumour) [13] in which an approximately 2-dimensional sheet of tissue could be scanned by NADH microfluorometry for localized areas of hypoxia.

The recording microfluorometer specially designed for recording NADH fluorescence from tissue surface has been described elsewhere [10]. The area seen by the photomultiplier was restricted to 8 μ diam. by placing a 1 mm diaphragm in the image plane. The animals bearing tumour were anesthetized by urethane. Respiratory gases were controlled by a gas mixer and administered by a mask placed over the animal's snout. The 366 nm excitation beam was focused on the tumour and the scanning of the tumour surface was allowed by displacements of the animal which was placed on the microscope stage.

Figure 1 illustrates a typical reaction to changes in the respiratory gas mixture in an oxygen responsive portion of the sandwich tumour. Time proceeds from left to right in Fig. 1 and fluorescence increase is indicated as an upward deflection. The baseline for the

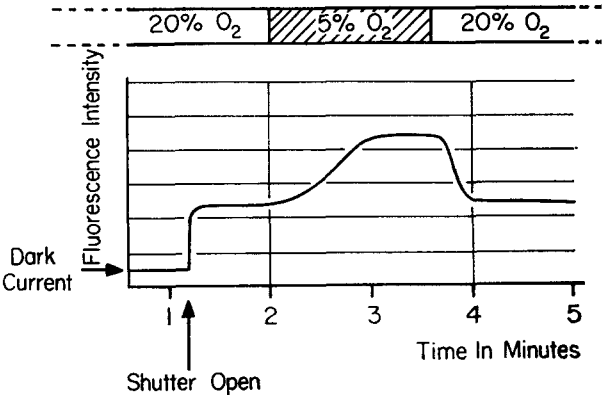

Fig. 1. Example of the response of the NADH fluorescence of the C_3H mouse mammary tumour to a decrease of the oxygen concentration of the inspiring air. The fluorescence signal doubles (this is calculated as a 100% increase) when the animal is made hypoxic, and returns rapidly to the normal value when rebreathing air. Exciting wavelength: 366 nm, fluorescence: 450 nm.

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†The work reported in this paper was undertaken during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer.
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unexposed photomultiplier is indicated in the initial portion of the trace and after 1·2 min of the recording the shutter is opened giving a normoxic value of tissue fluorescence. At 2 min the animal breathes 5% oxygen and almost immediately the fluorescence increases to the hypoxic level, approximately double the normoxic level, which is reached at 3 min and maintained 0·8 min when the animal is allowed to breathe again normal oxygen tension, diminishing the fluorescence to the normoxic level.

Figure 2 illustrates an experiment where a continuous scan was performed in normoxia along the line represented by the points. The scan was repeated in anoxia and calculations were made of the fluorescence increase in anoxia, compared to the normoxic conditions, obtained at the particular points which are circled. In the relatively open space between major blood vessels and in the region of small capillary a large fluorescence change (67%) was observed, similar to that obtained in Fig. 1. In other regions even though in the vicinity of blood vessels and branched points in the circulatory system the fluorescence was smaller, ranging from 20 down to 7%.

One interpretation of the results is that the areas which showed a smaller fluorescence increase had a greater degree of pyridine nucleotide reduction when the animal was breathing the normal oxygen tension and might

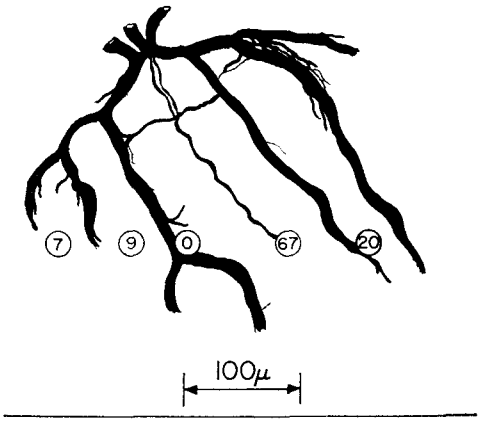

Fig. 2. Differences in the relative increase of the NADH fluorescence to a decrease of the oxygen concentration of the inspiring air (Fig. 1) for different locations in a sandwich tumour. The circles represent the measured areas; the figures in the circles give the percentage of fluorescence increase with 5% oxygen compared to the fluorescence with 20% oxygen for that location. The actual measurement of fluorescence in normoxia and anoxia at each point was performed as in Fig. 1 by continuous scan of each point in normoxia and anoxia.

therefore be considered hypoxic regions of the tumour.

In summary, these experiments show the feasibility of employing a scanning microfluorometer technique for identifying *in vivo* the existence and the location of hypoxic foci and may be of great usefulness in evaluating radiosensitivity in a quantitative relationship to the degree of intracellular hypoxia.

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E.O.R.T.C. News

THE FIRST meeting of the European Organization for Research on Treatment of Cancer (E.O.R.T.C.) Study Group on extracorporeal irradiation of the blood (ECI) was held in Brussels at the Institut Bordet on Saturday, 18th September 1971. Twelve members of the Group were present, as follows:

Dr. B. Rosengren (Göteborg)
Professor C. Parmentier (Villejuif)
Professor J. L. Binet (Paris)
Dr. R. Schaerer (Grenoble)
Dr. P. Stryckmans (Brussels)
Dr. Hagenbeck (Rijswijk)
Dr. Bock (Ulm)
Dr. Fröhlich (Ulm)
Dr. M. W. Hess (Bern)
Dr. E. O. Field (London)
Dr. J. A. Bullimore (Bristol)
Professor L. G. Lajtha (Manchester) (In the Chair).

Another five members, who were unable to attend the meeting, sent information by letter, as follows:

Dr. E. Weeke (Copenhagen)
Dr. S. A. Birkeland (Odense)
Dr. H. Theml (Munich)
Professor H. Cottier (Bern)
Professor T. A. J. Prankerd (London).

The discussion indicated that the use of ECI centred round two problem complexes: (1) leukaemia, and (2) immunodepression.

(1) *Leukaemia*

The interest is primarily in chronic lymphocytic leukaemia (CLL) in which several points of view were proposed, ranging from treating all patients of CLL with ECI, to selecting advanced cases with aplasia, or with highly elevated white cell counts, or resistant to chemotherapy. These were proposed by the members at Ulm, Paris and London, respectively. A proposal was also made that the acute lymphoblastic leukaemia and myeloid leukaemia also might benefit from ECI before chemotherapy, with the purpose of increasing the sensitivity to drugs by previous ECI treatment. The latter was proposed by Professor Binet in Paris. The Scandinavian Group have done CLL work, but their approach is being kept in abeyance until some reliable criterion can develop indicating those patients who would predictably benefit from ECI.

It was not possible to agree on a co-operative clinical trial by the whole Group because of their present divergence of views, but the Ulm and Munich Group are already collaborating on a clinical trial and the Paris and London people will yield sufficient patient material within the next two to three years so that a meaningful comparison will probably be possible.

(2) *Immunodepression*

The immunodepressant activity of ECI is being used at present in a co-operative trial in Scandinavia (Copenhagen, Odense, Göteborg), to assess its effectiveness as a supplementary treatment following kidney grafting. The pilot experiments indicated both feasibility and promise, and a random clinical trial has been started between these three centres. The results are followed by sequential analysis and apart from registering graft rejection times a number of other immunological and cell kinetic parameters are being recorded. This work overlaps, at least in interest, with the immunocytokinetic studies carried out at Brussels, Bern and Rijswijk, as well as the study of the effect of ECI on plasma proteins carried out in Grenoble. The kinetic studies are concerned with cell population changes particularly following lymphocytic mobilization, the end points being both peripheral count and lymph node architecture.

This first meeting was primarily for dissemination of information within the Group, and it was agreed that the data obtained so far could be tabulated by each centre, then duplicated to be sent out to all members of the Group for information. This tabulation will primarily refer to the leukaemia data since the clinical trial on immunodepression requires periodic up-dating of available information.

It was agreed that the next meeting of the Group will be in mid 1972.

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An Appreciation of Professor Hans Lettré (1908–1971)

ON 27 JULY 1971, Professor Dr. Hans Lettré, the Chairman of the local organizing committee for the 4th International Symposium on the Biological Characterization of Human Tumours, died suddenly at the age of 63 years.

Born in Wuppertal, he studied mathematics and chemistry in Munich and Göttingen. He worked with Professor A. Windaus in a long and productive study of the chemistry and physical chemistry of sterols and steriods including syntheses of cytotoxic derivatives (5, 6-seco-sterols, aza-steroids).

In 1939 he became director of the chemistry department of the "Rudolf Virchow" Hospital, Berlin. Here he developed the method of tissue culture as a test-system for chemical factors on cell growth and the Ehrlich ascites tumour as one of the first systems for anticancer drug screening. His work on the relation between chemical structure and antimitotic action of colchicine and its derivatives, which was started during this period, produced some highly active colchine analogues which still have clinical application.

He was appointed to take over the academic obligations of Professor Windaus in Göttingen in 1942 and, six years later, became director of the Institute for Experimental Cancer Research of the University of Heidelberg (which is now a part of the German Centre for Cancer Research), a post which he held until his death.

Between 1949 and 1971, some 500 papers and about 300 theses were published from the Institute. Of special interest are the publications on cytoxic purinyl-6-derivatives, among which purinyl-6-histamine is outstanding because of its selective cytoxicity against tumour cells *in vitro*.

Professor Lettré was widely sought as a lecturer and advisor and was the recipient of many honours including:

1943—Carl Duisberg Award of the German Chemical Society,
1949—National Award of the German Democratic Republic,
1966—Wilhelm Warner Award for Cancer Research.

He was a member of several international societies including the New York Academy of Sciences, the Accademia Tiberina, Rome, and the International Society for Cell Biology.

His co-workers, the many scientists who enjoyed the hospitality of his institute and his colleagues in the Co-ordinating Committee for Human Tumour Investigation will remember Professor Hans Lettré as a stimulating and generous friend.

The following papers, presented at the 4th International Symposium on the Biological Characterization of Human Tumours, stand as a tribute to his memory.

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Glucuronyltransferase Activity Towards Diethylstilbestrol in Rat and Hamster: Effect of Activators*

MARÍA GABALDÓN and TERESA LACOMBA

Servicio de Cancerología Experimental, Facultad de Medicina, Paseo al Mar, Valencia, Spain

Abstract—*The effect of Triton X-100, EDTA, ATP+UDP-N-acetylglucosamine (UDPAG) has been studied on the hepatic glucuronyltransferase activity towards diethylstilbestrol (DES) in hamster and rat. In hamster only EDTA does not modify the activity; Triton and ATP+UDPAG are activators and the activation produced is additive and of a similar magnitude. In rat only ATP+UDPAG produces an increase of activity, this effect disappearing when Triton is utilized with them. The activity is much higher in hamster than in rat in all the conditions of activation studied.*

Breakdown of UDPGA has been studied in rat in order to know whether the destruction of UDPGA by pyrophosphatase is a rate-limiting factor of glucuronidation in rat. Activity in rat does not increase in the cases where UDPGA breakdown is not complete, which suggests that the rate-limiting factor of glucuronidation in rat is the low content of enzyme.

INTRODUCTION

CHRONIC administration of DES† produces renal tumors in the male hamster but not in the rat [1]. Taking into account the fact that the only DES metabolite that has been described is DESGA, we have studied in previous works [2,3] the glucuronyltransferase activity towards DES in liver and kidney homogenates of rat and hamster in order to find some relation between DES metabolism and the production of renal tumors.

In vitro, glucuronyltransferase activity towards DES, both in liver and in kidney, is 5 to 6 times higher in hamster than in rat; in the latter animal the activity being very low in both organs. However, *in vivo* rat liver forms DESGA at a greater rate than would be expected from the results obtained by us *in vitro*. Rats administered intraperitoneally with 2 mg of DES excrete 40% of the dose as DESGA in the bile at 3 hr [4] and 67% at 24 hr [5]. There are no references concerning DESGA formation by hamster liver *in vivo*.

It is known that there is a pyrophosphatase that hydrolyzes UDPGA [6] and that the enzyme levels are much higher in rat than in other animals [7]. Pogell and Leloir [8] found that ATP and UDPAG were glucuronyltransferase activators and the authors explained this as being in part, a competition phenomenon of each of the substrates for the pyrophosphatase site, more UDPGA remaining intact for the conjugation reaction. EDTA inhibits UDPGA breakdown by pyrophosphatase [7,9,10,11] and a glucuronyltransferase activator results in the cases where UDPGA disappearance is the rate-limiting factor of the

Accepted 20 January 1972.

*Presented at the Fourth International Symposium on the Biological Characterization of Human Tumors, Heidelberg, April 21–23, 1971.

†The abbreviations used are: diethylstilbestrol, DES; diethylstilbestrol monoglucuronide, DESGA; uridine diphosphate glucuronic acid, UDPGA; adenosine triphosphate, ATP; uridine diphosphate-N-acetylglucosamine, UDPAG; ethylene diamine tetraacetic acid, EDTA; trichloroacetic acid, TCA.

reaction [12]; when this does not occur, EDTA acts as an inhibitor. Lueders and Kuff [13] and Winsnes [14] described the activator effect of Triton X-100 upon the glucuronyltransferase.

These observations raised the possibility that activities observed in unactivated preparations of rat could be misleading in terms of actual enzyme content. In the present work, glucuronyltransferase activity towards DES in hamster and rat liver homogenates has been studied in the presence of Triton X-100, ATP + UDPAG, the three together, and EDTA in order to ascertain the rate of activity in these two animals in different conditions of activation.

MATERIAL AND METHODS

Chemicals

DES, UDPGA, ATP, UDPAG and Triton X-100 were provided by Sigma Chemical Co.; tris(hydroxymethyl)aminomethane, (tris) and propylen glycol by Schuchardt; EDTA by Riedel de Häen; the other reagents were of analytical grade, the solvents were redistilled and diethyl ether was maintained free from peroxides by storage over ferrous sulfate and was freshly distilled before use.

Animals

Three-month old male Wistar rats and golden hamsters were used. The animals were sacrificed by cervical dislocation and the livers immediately removed and washed with 0·15 M KCl–0·32 mM $KHCO_3$ solution at 0°C; 15% homogenates were prepared with the same solution in a Potter-Elvhjem homogenizer.

Determination of glucuronyltransferase activity towards DES

The technique described by Gabaldón *et al.* [15] was utilized with the following modification: the reaction was stopped by addition of 5 ml of 20% TCA: ethanol (1:4) and the precipitate was separated by filtration 15 min later. The composition of the incubation mixture was as follows: tris, 35 mM; UDPGA, 0·42 mM; DES, 0·149 mM; $MgCl_2$, 10·5 mM; 3% v/v propylene glycol; 15% homogenate, 1 ml; final volume, 5 ml; pH, 7·3; DES was added to the incubation mixture dissolved in 60% propylene glycol. The concentrations of the activators in the incubation mixture were: Triton X-100, 0·05% v/v; ATP, 1 mM; UDPAG, 0·5 mM; EDTA, 10 mM. In the assays with EDTA, $MgCl_2$ was omitted and 50 mM phosphate buffer in the incubation mixture was used instead of tris buffer. In the assays with Triton + ATP + UDPAG in the hamster, the DES concentration utilized was twice that of the standard incubation mixture. DES was added to the incubation mixture dissolved in 75% propylen glycol. The following modification was introduced with respect to the technique previously described [15] for the determination of DES in the incubation mixture: 7·5 ml of a freshly prepared 0·67% $NaHCO_3$ was added to an aliquot of 2·5 ml from the filtrate.

Breakdown of UDPGA

Standard incubation mixtures with rat liver homogenates were incubated in the presence and absence of UDPGA for 20 min at 37°C and the technique described for the determination of glucuronyltransferase activity towards DES was followed. DES consumed in the absence of UDPGA was subtracted from DES consumed in the presence of UDPGA.

15% liver homogenates of the same rats utilized in the above paragraph were incubated with tris buffer and $MgCl_2$ in the presence (PU) and absence (AU) of UDPGA for 20 min at 37°C. The mixtures were then heated at 100°C for 10 min, cooled and filtered. 3·16% homogenates of hamster liver were made in each of the filtrates proceeding from PU and AU. The UDPGA content in the homogenate made in PU was what remained after rat liver pyrophosphatase action; 0·25 ml of a 800 μg/ml DES solution in propylene glycol 60% were added to 4·75 ml of each of the homogenates. The mixtures were incubated for 20 min at 37°C and the technique described for the determination of glucuronyltransferase activity was followed. DES consumed in the absence of UDPGA was subtracted from DES consumed in the presence of UDPGA.

RESULTS

Table 1 shows the effect of activators on glucuronyltransferase activity towards DES in rat. In the absence of UDPGA none of the activators results in statistically significant differences of activity; in the presence of UDPGA only ATP + UDPAG produces an increase of activity and this increase disappears when Triton is utilized with them. The differences of DES consumption in the presence and absence of UDPGA are not statistically significant in the case of the controls and the assays with EDTA, and are significant in the rest of the cases. With UDPGA concentrations up to 4·2 mM, Triton does not produce any activation relative to the control.

Table 1. *Hepatic glucuronyltransferase activity towards DES in rat. Effect of activators*

Activator	Without UDPGA	*P**	With UDPGA	*P*
Control (6)†	8·7 ± 12·7‡		27·5 ± 14·5	
Triton (5)	10·7 ± 8·2	N.S.	39·2 ± 10·2	N.S.
ATP + UDPAG (9)	18·5 ± 21·5	N.S.	94·7 ± 24·7	< 0·001
Triton + ATP + UDPAG (7)	7·5 ± 15·5	N.S.	38·0 ± 15·2	N.S.
EDTA (5)	17·5 ± 16·5	N.S.	46·0 ± 21·7	N.S.

*Level of significance, Student *t* test; $P > 0{\cdot}005$ = N.S.; *P* value compared with the control.
†Numbers in parenthesis indicate number of animals used.
‡Values are given in mμmoles DES consumed/100 mg fresh liver/90 min. Results are given as the mean ± S.D.

Table 2. *Hepatic glucuronyltransferase activity towards DES in hamster. Effect of activators*

Activator	Without UDPGA	*P**	With UDPGA	*P*
Control (6)†	46·5 ± 25·0‡		193·7 ± 21·2	
Triton (8)	24·7 ± 10·5	N.S.	431·2 ± 28·2	< 0·001
ATP + UDPAG (6)	54·7 ± 16·0	N.S.	434·5 ± 29·2	< 0·001
Triton + ATP + UDPAG (5)	110·0 ± 28·5	N.S.	738·7 ± 30·7	< 0·001
Control (8)	37·2 ± 26·7		123·2 ± 25·2	
EDTA (9)	42·0 ± 17·0	N.S.	151·5 ± 17·5	N.S.

*Level of significance, Student *t* test; $P > 0{\cdot}005$ = N.S.
†Numbers in parenthesis indicate number of animals used.
‡Values are given in mμmoles DES consumed/100 mg fresh liver/90 min. Results are given as the mean ± S.D.

Table 2 shows the effect of activators on glucuronyltransferase activity towards DES in hamster. In the presence of UDPGA all the activators, except EDTA, cause statistically significant increases of activity while in its absence there are no significant variations. The differences of DES consumed in the presence and absence of UDPGA are statistically significant in all the cases. The activation by Triton and ATP + UDPAG is additive and of a similar magnitude. Controls performed with phosphate buffer in the absence of $MgCl_2$ give values lower than the ones performed with tris buffer in the presence of $MgCl_2$, the differences being statistically significant in the presence of UDPGA. Mg^{2+} can act as activator or inhibitor depending on the concentration [16]. Probably in our case the differences of activity between the controls are due to the absence of $MgCl_2$ instead of to the buffer.

Five assays were performed in order to ascertain the intensity of UDPGA breakdown by rat liver homogenates. The differences of DES consumed in the presence and absence of UDPGA by rat and hamster liver homogenates are shown in Table 3; in the case of the rat the concentration of UDPGA was 0·42 mM and in the case of the hamster what remained after rat liver pyrophosphatase action. In two experiments DES consumption by hamster liver homogenates was negligible which indicates that UDPGA was completely destroyed by rat liver pyrophosphatase; however, in the other three cases, the breakdown of UDPGA

Table 3. *UDPGA breakdown by rat liver homogenates*

	Differences of DES consumed in the presence and absence of UDPGA		
	Complete breakdown	Partial breakdown	*P**
Rat	27·0 ± 8·0† (2)‡	30·0 ± 7·0 (3)	< 0·005
Hamster	5·0 ± 2·5 (2)	87·0 ± 12·5 (3)	

*Level of significance, Student *t* test;
†Numbers in parenthesis indicate number of animals used.
‡Values are given in mμ moles DES consumed/100 mg fresh liver/20 min Results are given as the mean ± S.D.

by rat liver pyrophosphatase was not complete because there was DES consumption by hamster liver homogenates.

DISCUSSION

The rat does not show more activity when UDPGA has been only partially destroyed than when UDPGA breakdown has been complete. If the low activity found in the rat was due exclusively to a UDPGA deficiency, one could expect a large increase in the presence of EDTA, which inhibits almost completely UDPGA breakdown by pyrophosphatase. In our case, EDTA had negligible effect on the glucuronyltransferase activity of the rat. The same was found by Huttunen and Miettinen [11] with homogenates of young rats although these authors pointed out that the response produced by EDTA was somewhat dependent on the age and sex of the animals.

Winsnes [14] found that activation of glucuronyltransferase by preincubation, detergents and UDPAG was nonadditive, of a similar magnitude and had the same effect on the UDPGA saturation curves; for these reasons he supposed that UDPAG, being a substrate analogue, might act as an allosteric activator of the enzyme, which would result in the same unmasking of the active site as that obtained with detergents and preincubation. This explanation does not agree with our results because we have found that in hamster, activation by Triton and ATP+UDPAG is additive, while in rat, activation by ATP+UDPAG disappears in the presence of Triton. The effect of Triton seems to depend on the substrate acceptor and the species. Winsnes [14] found that glucuronyltransferase of mouse liver was not activated by Triton when the substrate acceptor was *o*-aminophenol although the same activity towards other acceptors was highly increased.

All the results obtained seem to indicate that the actual content of hepatic glucuronyltransferase towards DES is much greater in hamster than in rat because (1) in 20 min the hamster liver conjugates 3 times more DES than the rat liver although UDPGA levels in the case of hamster are lower than in the case of the rat (in the preincubation with rat liver UDPGA disappears partially by pyrophosphatase action) and (2) in all the conditions of activation studied, glucuronyltransferase activity is much higher in hamster than in rat.

The prolonged treatment with estrogens decreases glucuronyltransferase [17]. In the case of the hamster the decrease of activity with DES treatment is gradual and after 7 months the activity even reaches approximately 50% of the initial level, an amount considerably higher than that of the non-treated rat [3]. The major interest of our work focuses on the possibility that renal tumor production in hamster by DES may be due to a deficiency in DES glucuronidation in relation to rat which is not affected by DES treatment. From the results so far obtained, we can say that this does not seem to be the cause of renal tumor production because glucuronyltransferase towards DES is much greater in hamster, treated and non-treated with DES, than in rat. Another explanation lay in the possible tumorigenic activity of DESGA, the main metabolite of DES, but this possibility is excluded as we found that chronic treatment with DESGA does not produce renal tumors in hamster [3]. All this seems to indicate that hamster kidney is much more responsive to DES than that of the rat as it is affected by lower DES levels. The mechanism by which hamster kidney has such a peculiar response to estrogens is now under study.

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Clinical and Experimental Tumors of the Kidney in Tissue Culture and in the Chick Embryo*

J. LEIGHTON, S. MANSUKHANI and G. H. FETTERMAN

The Departments of Pathology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129, *and The Children's Hospital, Pittsburgh, Pennsylvania* 15213, *U.S.A.*

Abstract—*A brief report is presented on several lines of study involving solid tumors of the kidney. Wilms' tumors, either immediately after surgery or after freezing preservation, have been transplanted successfully to the chorioallantois of the chick, in combination with normal chick embryonic tissues. The transplanted tumors grew as nodules of anaplastic cells and as neoplastic tubules with various degrees of differentiation. Applying the techniques of renal microdissection to surgical specimens of Wilms' tumors, we have seen details of tubular structure and branching. A renal cell carcinoma, MDCK, derived from normal dog kidney, grew in culture with one of two patterns of polarization, depending upon the microenvironment of the cells. The implications of these studies for the treatment of clinical cancer is considered.*

THIS IS a progress report on two experimental studies using solid tumors of the kidney. Although the two projects are progressing independently at this stage of their development, the direction of each is such that they will meet and should partially illuminate one another.

In one study, we are concerned with the formation of tumor nodules on the chorioallantoic membrane of the chick embryo following the topical inoculation of fragments of Wilms' tumor. Tumor is inoculated immediately after the surgical specimen is obtained; in addition, fragments of the specimen are inoculated following prolonged storage over liquid nitrogen. We have identified the conditions in which proliferating transplants of Wilms' tumors grow on the chorioallantois with histotypic fidelity. In a preliminary experiment typical patterns of Wilms' tumor have also been produced when the inoculum was cultured in a three-dimensional matrix of collagen-coated cellulose sponge.

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In the second project we are using an experimental renal cell carcinoma, MDCK, to study the significance of polarization of epithelial cells in tumor nodules. The cell line MDCK, derived from normal dog kidney, grows as a papillary adenocarcinoma either in the chick embryo or in three-dimensional tissue culture [1]. *In vitro* the tumor cells appear in cystic groups, with the columnar epithelium polarized in one of two opposite arrangements, depending on the microenvironment of the cells.

WILMS' TUMORS

The tumors of 10 patients have been studied. In each case, slices of tumor were removed from the surgical specimen immediately after excision, and placed in a medium consisting of 30% calf serum in Eagle's Minimal Essential Medium supplemented with antibiotics. Each donor tumor was then divided into three portions.

One portion was used for transplantation to the chorioallantois (CAM) of chick embryos ranging in age from 8 to 11 days. The minced tumor was inoculated alone, or in combination with a fine mince of normal chick embryonic

tissues taken from embryos the same age as the recipient. The mesonephric region, heart, or liver was used. Embryos were harvested on the 15th and 18th days of incubation. The second portion of the tumor was implanted on collagen-coated cellulose sponges either alone or in combination with embryonic tissues. The third portion was suspended in 10% glycerine, or 5% DMSO, divided into 1 ml aliquots and frozen preserved over liquid nitrogen. We processed 7 of the tumors immediately on receiving them from the operating room. Two were stored in medium overnight at 4°C. before processing, and one was stored for 48 hr.

The presence of a mince of embryonic tissue was essential for consistently successful transplantation of Wilms' tumors to the CAM. When the original tumor alone was inoculated, viable tumor nodules in the CAM were small and appeared irregularly. When the inoculum consisted of tumor and chick embryonic tissue, neoplastic growth was abundant and reproduced the components of the tumor as seen in the original surgical specimen, namely masses of anaplastic cells, tumor stroma, and neoplastic tubules in various stages of differentiation. Anaplastic tumors retained their qualities on transplantation, as did tumors containing many neoplastic tubules. Marked angiogenesis in the stroma of the CAM was also noted near some tumor transplants. Of the three embryonic tissues used, liver was the most suitable. It has no cellular components that can be confused with elements of the tumor; it is morphologically uniform, and relatively large. The total mass of tumor growth was slightly greater in the presence of liver than when the other embryonic tissues were used.

Frozen–thawed tumor combined with fresh embryonic tissue was inoculated on the CAM of 10-day old embryos. These inocula gave rise consistently to multiple histotypic nodules of Wilms' tumor growth in six of the seven cases studied thus far (see Table 1). Figures 1, 2 and 3 illustrate the histologic appearance of chorioallantoic transplants of thawed tumors from three different patients. In all three cases the tumor was combined with tissue from the mesonephric region. The histologic features of Wilms' tumors, masses of anaplastic cells and neoplastic tubules, are easily recognized.

When Wilms' tumor alone was inoculated in collagen-coated cellulose sponge, the outgrowth consisted primarily of a dense meshwork of large fibroblast-like cells with a scattering of epithelial tubules. In a recent experiment a specimen of frozen–thawed tumor (case 5) was combined with minced embryonic liver. Organoid tumor growth was found in all four cultures after six days. Figure 4 is a field from one of these cultures. A comparison of Fig. 3 and 4 is appropriate. They represent growth from a single tumor specimen (case 5) after thawing, combined with chick embryo tissue and inoculated in the embryo (Fig. 3) and in collagen-coated cellulose sponge (Fig. 4). The pattern of growth is similar; anaplastic cells appear to be forming neoplastic tubules.

Table 1. Wilms' tumors transplanted to the chorioallantois of the chick embryo

	Fresh tumor			Frozen–thawed tumor		
Case	Eggs inoculated	Viable at harvest	Pre-op therapy	Eggs inoculated	Viable at harvest	Successfully recovered
1*	22	18	None	None	None	—
1a*	12	12	Dactino and radiation	12	12	Yes
2	20	9	Dactino	30	11	No
3	36	32	None	30	15	Yes
4	27	22	None	23	11	Yes
5	33	31	Tumor perfused with Dactino	23	10	Yes
6*	14	12	Radiation and Dactino	22	14	Yes
7	30	12	Dactino	30	18	Under study
8	40	34	None	30	24	Under study
9	40	37	None	19	13	Under study
10	30	28	None	30	20	Yes

*Cases # 1 and 1a came from the same patient at different times. The inoculum for # 1 was from a metastasis in an abdominal lymph node. Specimen # 1a was from a metastasis in the lung. The specimen from case # 6 was also a lung metastasis. In all other cases the specimen was from the primary tumor in the kidney.

Fig. 5. Mosaic photomicrographs of many irregularly branching, grotesque, neoplastic tubules dissected from Wilms' tumor. Many of the protuberances represent diverticula; many represent branches which have become broken. × 200.

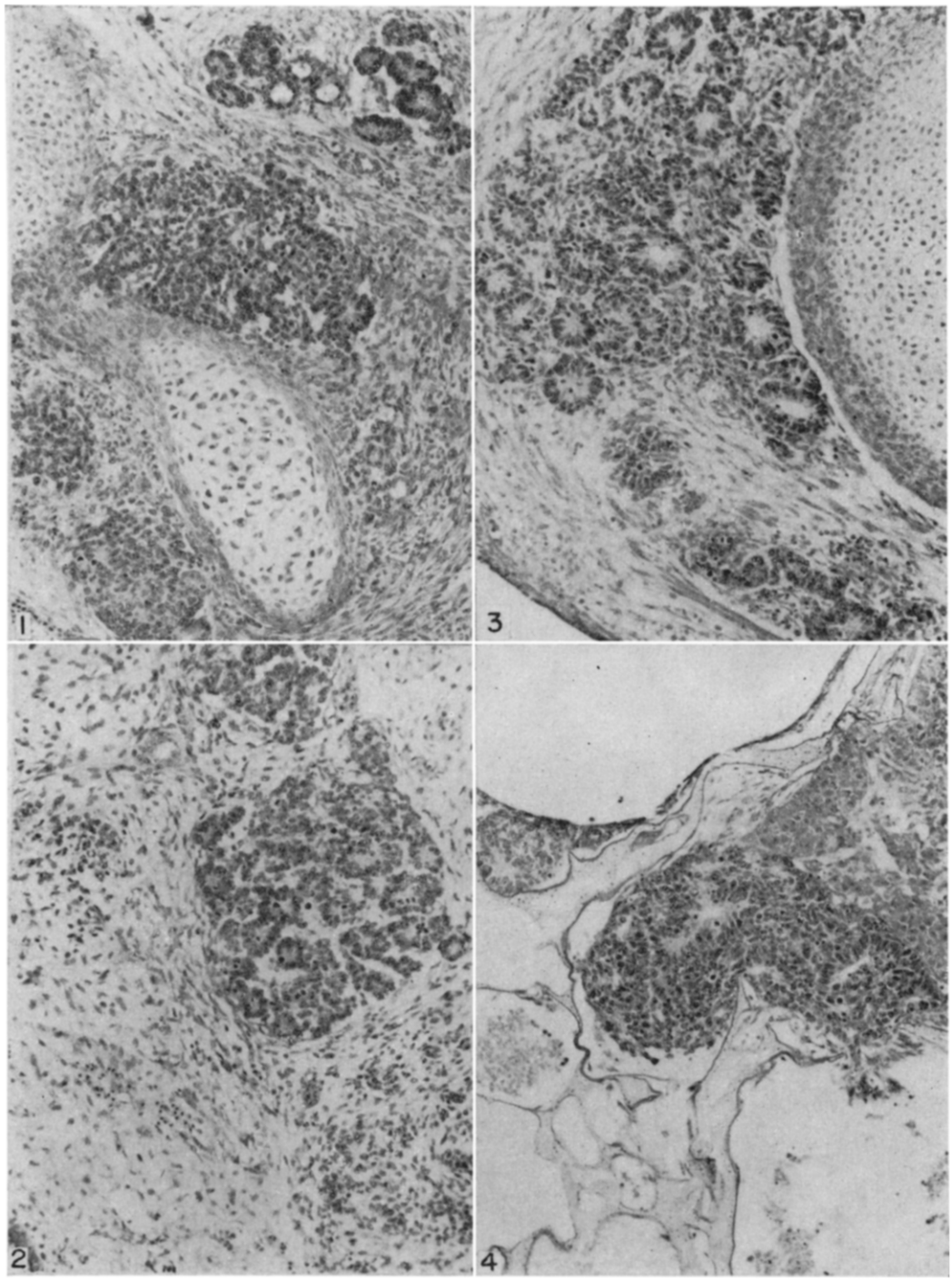

Fig. 1. *Histologic section of frozen-thawed Wilms' tumor (case* 3*) inoculated in combination with a mince of the mesonephric region, on the chorioallantois of a* 10*-day old embryo, and examined* 8 *days later. A group of differentiating tubules is seen at the top. Near the center of the field, adjacent to cartilage, a mass of anaplastic cells with many mitotic figures appears to be forming tubules. Two other small foci of tumor are seen in the lower left, below the cartilage. Hematoxylin and eosin,* × 150.

Fig. 2. *Histologic section of frozen-thawed Wilms' tumor (case* 4*) inoculated in combination with a mince of the mesonepric region in a* 10*-day old embryo, and examined* 8 *days later. Part of a collection of irregular neoplastic tubules is seen at the top of the figure. Just below it is a complete nodule of tumor tubules. These two foci of tumor are separated by a narrow band of connective tissue. A single pale staining chick embryonic kidney tubule with a distinct lumen is seen on cross-section just to the left of this connective tissue band. Hematoxylin and eosin,* × 150.

Fig. 3. *Histologic section of frozen-thawed Wilms' tumor (case* 5*) on the chorioallantois in combination with embryonic mesonephric region. Tumor appears* 5 *days after inoculation as a crescent shaped mass of many hyperchromatic neoplastic tubules containing many mitoses. A large mass of embryonic cartilage is seen on the right side of the figure. Hematoxylin and eosin,* × 150.

Fig. 4. *Histologic section of frozen-thawed Wilms' tumor (case* 5*) grown with embryonic liver in tissue culture on a matrix of collagen-coated cellulose sponge. In the center of the field a mass of anaplastic tumor cells with numerous mitoses appears to be forming many neoplastic tubules. The tumor tissue is in direct contact with pale staining embryonic liver in the upper right corner of the figure . Hematoxylin and eosin,* × 150.

(to face p. 282)

Refrigeration of excised tumors overnight and longer had no obvious deleterious effect on the ability of these tumors to grow on the CAM. Slices of tumor from cases 3 and 4 were refrigerated overnight. A portion of the tissue from case 5 was processed immediately, but another portion was stored in the refrigerator for 48 hr before the experimental procedure was conducted. The growth from fresh and from refrigerated tumor was indistinguishable. Five of our 10 patients received therapy before operation, actinomycin D either alone or in combination with radiation. The growth of the treated and the untreated tumors was indistinguishable. Case 5 is of particular interest since the tumor in this patient had been perfused with 300 μg of actinomycin D, administered directly through the renal artery, on the day before operation.

In a related study the structure of the neoplastic tubules in surgical specimens of Wilms' tumors were examined with the methods of renal microdissection described by Oliver [2, 3]. Complex branching tubular structures have been isolated from samples of three tumors. The diameters of the tubules varied widely in any one tubular complex, and large numbers of budding protrusions, resembling diverticula, have been found. Figure 5, part of the microdissection of one tumor, illustrates an elaborate branching pattern of neoplastic tubules. The lumens, as well as the lining epithelium, are easily identified in many parts of the structure. Although renal microdissection has been used extensively in the past to delineate normal renal structure and diseased nephrons [3], to the best of our knowledge this is the first report of its use in the study of Wilms' tumors.

MDCK

Tissue culture cell line, MDCK, grew on the chorioallantois when a dense suspension, one or two drops of a 50 % packed cell volume, was inoculated topically. When the suspension was MDCK alone, growth consisted mainly of a monolayer of columnar epithelium confined to the surface of the CAM, and replacing the chorionic epithelium of the membrane. Such takes occurred in about 1/3 to 1/2 of the inoculated membranes. On the other hand, when the inoculum consisted of a combination of MDCK and minced embryonic tissue, vascularized nodules of papillary adenocarcinoma were found regularly in the mesodermal part of the CAM.

MDCK cultured *in vitro* expressed many morphologic features of papillary adenocarcinoma, and also had qualities of renal tubular transport [1]. In these two capacities, as carcinoma and as a kind of renal tubular epithelium, MDCK appeared as a polarized columnar epithelium. In stock cultures, MDCK appeared as a monolayer of epithelium interspersed with numerous multicellular hemicysts or blisters. In time-lapse movie studies we have found that these blisters became progressively larger as they became distended with fluid. They then abruptly collapsed. On electron microscopy, microvilli were found on the outer surface of the cells, the surface bathed by the tissue culture medium.

The cells of the monolayer were readily suspended with EDTA–trypsin for 30 min at 36°C. When dispersed cells were resuspended in fresh medium, and incubated in a bottle placed in a roller drum turning at 1 rev/min, many cells remained in suspension. After one day, the cells in suspension were almost all in compact aggregates. When such aggregates were continued in suspension for 4 or 5 days many became distended cysts, that is cystic papillary aggregates in suspension. On electron microscopic study the walls of the cysts were seen to consist of a polarized epithelium with the mircrovilli on the outer convex surface of the cysts. On the other hand, when one-day old compact aggregates were placed in a fibrin clot, prepared by combining heparinized chicken plasma and chick embryo extract, glandular cystic structures formed in a few days. Here the microvilli lined the concave lumens of the cysts. When compact aggregates were inoculated into the substance of collagen-coated cellulose sponge, they also became glandular cysts. Current studies are concerned with obtaining a series of clonal isolates of MDCK in order to determine whether the two patterns of polarized growth represent a phenotypic response to the microenvironment, or whether they are genetically determined.

DISCUSSION

The use of embryonic tissue as an aid in the transplantation of tumor tissue has a long history [4, 5]. Our experience with Wilms' tumors and with renal cell line MDCK confirms that the presence of embryonic tissue as a component of the inoculum aids in the transplantation of heterologous tumors to the chorioallantois. Although the mechanism is not understood, the addition of embryonic tissue to a tumor inoculum constitutes a practical laboratory tool with which to study kidney tumor transplants on the CAM.

Two essential requisites must be met for laboratory methods to be of use in the bioassay of individual clinical tumors [6]. First, cancers must grow in the laboratory with the same histotypic features as the original surgical specimen. Also the laboratory must have available many aliquots of preserved viable tumor from each patient for successive follow-up studies. Our work on a series of 10 specimens, though still incomplete, indicates that these requisites have been fulfilled for Wilms' tumors.

Upon completion of the present study, we plan to compare the tumors we have stored in a frozen state in terms of sensitivity to chemical and to physical agents, initially to actinomycin D and to hyperthermia (42°C). We want to determine whether distinct differences appear in the response of tumors taken from different patients. Histopathologic sections and autoradiography will be used to evaluate the responses, which may be of two types. One may be an inhibition of tumor growth. Another may be a qualitative change in parenchymal organization, or in the supporting connective tissues.

Fundamental questions of tumor differentiation and morphogenesis are raised by examination of Fig. 1–4. When one sees nodules of tumor consisting of anaplastic cells with poorly defined tubules and nearby nodules of well formed distinct tubules, one assumes that the first arrangement is the antecedent of the second. With adequate laboratory methods the assumption can be examined critically, and alternative possibilities considered. In such studies where differentiation is probably involved, it is convenient to have available various means of morphologic study. We have at one end electron microscopy for important ultrastructural detail, and at the other microdissection to observe the formation, development, size, connections, and structure of these complex organoid tubules (Fig. 5). Preliminary observations with microdissection demonstrate the inadequacy of routine histopathology as a means of studying the interrelationships of the various neoplastic components of a Wilms' tumor. With microdissection we found that the tumor cells are arranged as long, highly convoluted tubular structures with multiple branches and complex globular protrusions along their course. A proper appreciation of the biologic relationship of nodules of anaplastic cells to adjacent, well formed complexes of tubules, as seen for example in Fig. 1, requires an understanding of the spatial relationship of these components taking into account the serpentine configuration of these structures. The frequent prominent diverticula warrant further study to determine whether they are centers of new growth, or foci of non-proliferation or maturation. Reliable information on these small structures will best be obtained using microdissection, histochemistry, and autoradiography. Wilms' tumors growing with typical morphology in the chick or in matrix cultures can be treated with pharmacologic agents and labelled isotopes and easily subjected to microdissection analysis.

Clinical renal cell carcinoma may possibly be studied with the same procedures we have used for the Wilms' tumors. Recently, following the acquisition of the 10 cases of Wilms' tumors summarized in Table 1, we have initiated a study of renal cell carcinoma. Three specimens have been prepared. At this writing histologic sections are available on only one part of the first experiment, in which fresh tumor and embryonic liver or heart were mixed and inoculated on the chorioallantois. In these sections, we have seen many nodules consisting of an intimate mixture of embryonic tissue and proliferating, vascularized, typical renal cell carcinoma.

Our cell line of dog kidney origin provides a convenient laboratory model for exploring some aspects of renal cell carcinoma that cannot yet be studied with clinical material. The two patterns of polarization expressed by this cell line may have implications for cancer therapy, expecially if one configuration of polarization is consistently more sensitive to cytotoxic chemicals than the other. In that event, it will be necessary to develop new agents to act primarily on tumor morphogenesis, agents that favor the growth of tissue configurations with maximal sensitivity to therapy.

We believe that the solid tumors of the human kidney, Wilms' tumors and renal cell carcinoma, are excellent potential candidates for studying the effects of chemical agents and other modes of therapy on the interrelated mechanisms of growth, malignancy, morphogenesis, and differentiation. Cells of kidney tubular origin, whether normal or neoplastic, provide an array of convenient morphologic and biochemical markers with which to monitor some of these interrelationships. Since we are expanding our capacity for experimentation on solid tumors of the human kidney on the one hand, and exploring fundamental aspects of kidney tumor morphogenesis with a related tissue culture cell line on the other, the two lines of study are becoming progressively interrelated.

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Diagnostic Cytology of Urinary Tract Carcinoma

A Review of Experience with Spontaneous and Carcinogen Induced Tumors in Man*

MYRON R. MELAMED

Attending Pathologist and Acting Chief, Cytology Service, Memorial Hospital for Cancer and Allied Diseases, N.Y.C., U.S.A.

Abstract—*Cytologic examination of the urinary sediment provides a very sensitive index of epithelial changes along the urinary tract. It is useful as a clinical tool to find early and occult carcinomas, and as a research tool to study the pathogenesis of human bladder carcinoma. From long term studies of industrial workers exposed to a bladder carcinogen (para-aminobiphenyl) and others with no known carcinogen exposure, a tentative outline of carcinogenesis in the bladder is proposed.*

URINALYSIS is the most ancient of medical laboratory tests, and microscopy of the cellular sediment has been the subject of much study almost since the development of the compound microscope. Yet only within the last two decades have exfoliated cancer cells been recognized in specimens of urine, and their significance understood.

The desquamated epithelial cells that are present in the urine represent an aliquot, a sample of the cells in the epithelium surfacing the urinary tract. They are a sensitive index of the cellular abnormalities that characterize the earliest carcinomas, carcinoma *in situ* [1–3]. Because of this, cytologic examinations of the urinary sediment are useful both as a clinical tool to find and diagnose occult carcinomas of the urinary tract, and as a research tool to follow the pathogenesis of human bladder carcinoma.

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CYTOLOGIC MORPHOLOGY

Normal

The normal urinary bladder epithelium has several layers of fairly uniform cells. Though there is cytoplasmic maturation in the superficial cellular layer, the nuclei of cells in all layers are quite regular, and very much alike. The cells that are shed from this normal epithelium are very sparse. One of the reasons why cytologic examination of the urine is such an excellent way to detect early bladder cancer is because there is a preferential sampling of cells from areas of carcinoma; large numbers of cells are shed from even very tiny carcinomas, while few cells are shed from the entire remaining normal epithelium. This is due partly to the more active proliferation of cancer cells compared with normal epithelium or with well-differentiated tumors (papillomas). It is due also to a loss of intercellular cohesion, fewer desmosomes and an absence of tight junctions and complex folding of surface membranes between the cancer cells [4].

Normal epithelial cells in the urinary sediment are characterized by smoothly contoured,

round or ovoid nuclei with delicate chromatin and a distinct nuclear membrane (Fig. 1). Nucleoli often are visible, and with some inflammatory reactions they may be prominent. Cytoplasm is variable in amount, depending on the preservation of the cells, and normally lightly eosinophilic. With inflammation, cytoplasmic staining is more intense.

Invasive carcinoma

Carcinoma of the bladder is made up of cells with enlarged, irregular nuclei that are hyperchromatic and appear to contain an increased amount of chromatinic material. They are variable in size and shape, often strikingly so, with varying amounts of cytoplasm that may or may not stain deeply eosinophilic. The cells appear in small groups or clusters as well as singly (Fig. 2). Invasive carcinomas are characterized by ulceration and inflammation, and this is manifested in cytologic preparations by leukocytes and blood in the background.

Papilloma

Papillary tumors of the bladder, and elsewhere in the urinary tract, are very common. The benign papilloma is formed of cytologically uniform and benign epithelium on a fibrovascular stalk (Fig. 3). Cells shed from this kind of tumor may be more abundant than the normal mucosa; they tend to be more elongate often, and may present in groups or clusters rather than as single cells. But they are not identifiable as malignant cells, and in a cytologic specimen it may not be possible to distinguish this benign tumor from normal.

Papillary carcinoma

Papillary carcinoma, on the other hand, is composed of malignant epithelial cells on a fibo-vascular core (Fig. 4). Epithelium is usually disorderly, and there is variation in cellular and nuclear size, configuration and staining characteristics. Cells with abnormally large and hyperchromatic nuclei are present. It is not uncommon to find areas of carcinoma within a papilloma. If there is no invasive carcinoma, we can then refer to this tumor as papilloma with carcinoma *in situ*.

Papillary carcinomas shed recognizable cancer cells in the urine, as well as benign epithelial cells, and they can readily be diagnosed by cytology. From a practical point of view, the cytologic examination of urine becomes a very useful way to identify papillomas that have areas of carcinoma *in situ*. We have seen instances in which initial random sections of a papillary tumor show only papilloma, but because of cytologic evidence of carcinoma in the urine more extensive study of additional tissue is carried out and carcinoma or carcinoma *in situ* demonstrated in the tumor. Thus, cytologic examination of the urine has become very useful to the urologist to tell him beforehand whether a papillary tumor is benign, or whether it is formed in part or wholly of carcinoma.

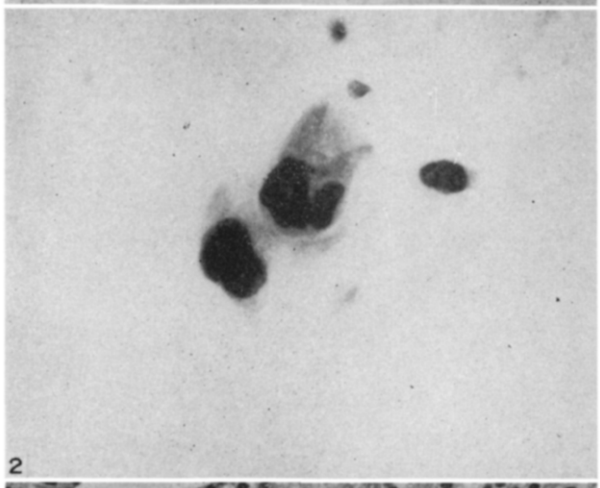
CYTOLOGIC OBSERVATIONS ON THE PATHOGENESIS OF BLADDER CARCINOMA

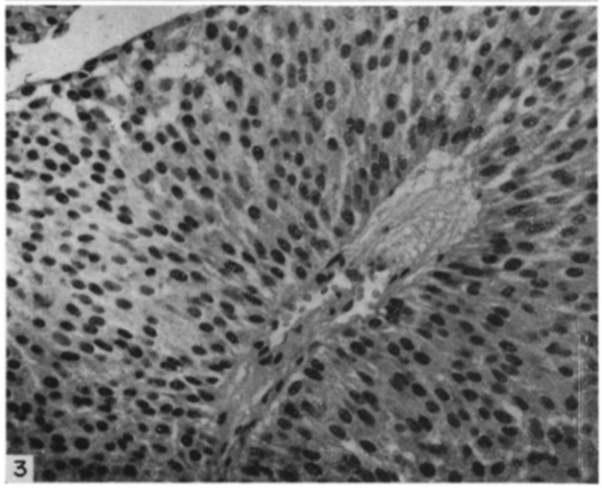
The greatest value of diagnostic cytology has been in detecting very early or very localized carcinomas, not only in the bladder but in ureters and renal pelvis. Obtaining and preparing specimens is quite simple, and simple for the patient, the major requirement being a high level of skill and experience on the part of the pathologist who must interpret the prepared slides. We have used cytology to follow high risk populations, that is, individuals with a high probability of developing bladder carcinoma, and it has given us considerable information about early stages in the pathogenesis of this disease.

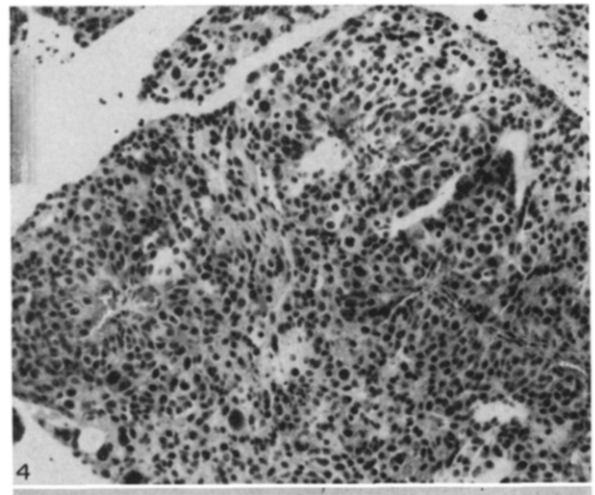
Industrial exposure to para-aminobiphenyl

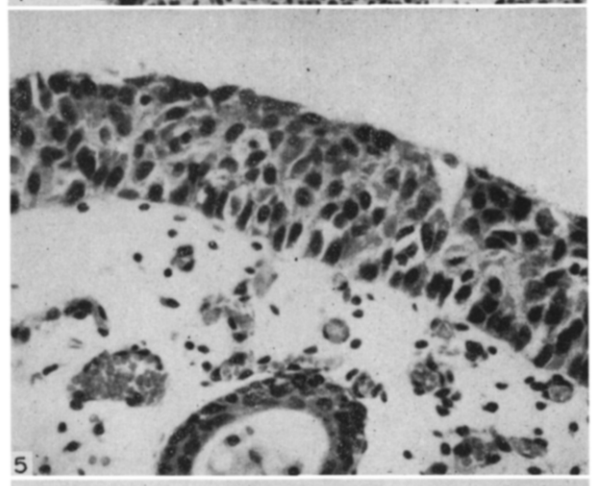
Some of our most informative studies began with a group of industrial workers in 1957, and they are still in progress today, almost 15 years later. Just 3 years before we began, in 1954, para-aminobiphenyl, a chemical that these men were working with, was shown to produce bladder cancers in dogs [5]. In 1955, the first cases in man were reported following exposure to this chemical [6]. At that time, all production of the chemical was terminated, and a careful clinical and laboratory study of the men at risk was begun.

During the succeeding 14 years, we have examined nearly 3000 cytologic specimens of urine from 541 men, some for over periods of time ranging up to 10 years or longer. Eighty-six men have had suspicious or positive cytology, either on initial examination, or subsequently appearing during the years under our surveillance, and so far, 43 of them have developed histologically confirmed carcinoma. The first event associated with exposure to the chemical, or following it, was transient dysuria. Though this was usually brief, reported by only a few of the men, and appeared innocuous clinically,









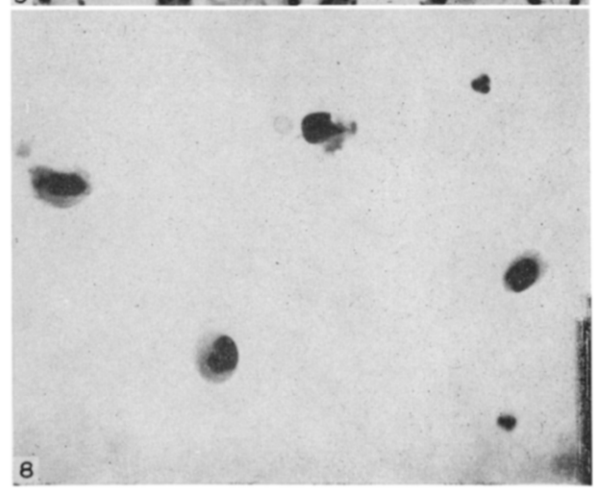


Fig. 1. *Normal, benign transitional epithelial cells in a cytologic preparation of urinary sediment. Papanicolaou stain.* × 560.

Fig. 2. *Desquamated cancer cells in the urinary sediment from a patient with invasive bladder carcinoma. The cells are large and bizarre with hyperchromatic, enlarged and irregular nuclei. Papanicolaou stain.* × 560.

Fig. 3. *Benign papilloma of bladder. The epithelium is composed of uniform, benign epithelial cells in orderly arrangement. When shed into the urinary sediment these cells are difficult to distinguish from normal bladder epithelial cells. H & E* × 350.

Fig. 4. *Papillary carcinoma of bladder. This tumor is composed of malignant epithelial cells on a fibro-vacuolar core, and desquamated epithelial cells in the urinary sediment have abnormally hyperchromatic, enlarged and irregular nuclei. H & E* × 140.

Fig. 5. *Carcinoma* in situ *of urinary bladder. The epithelial cells have enlarged, hyperchromatic, irregular nuclei. There is congestion and edema of the underlying stroma. H & E* × 140.

Fig. 8. *Cytology of carcinoma* in situ, *showing the relative uniformity of small malignant cells. Compare with Fig.* 2. *Papanicolaou* × 560.

(to face p. 288)

in retrospect it probably marked a time of significant injury to the epithelium. After exposure to para-aminobiphenyl had stopped and we began to examine many of these men we found carcinomas in some, but in others no abnormality was found either clinically, cystoscopically or cytologically. However, some irreversible change had occurred, because without further exposure to the chemical many of the men did ultimately develop bladder cancer.

Cytologic diagnosis of clinically occult carcinoma

Our first clue to the nature of carcinogenesis in the bladder came as we found cancer cells in the urine of some men who had no apparent tumors in the bladder. Efforts to confirm the cytologic diagnosis of carcinoma were unsuccessful because no abnormality was found by cystoscopy, and no biopsies were taken. Cytologic evidence of cancer was confirmed on repeat examinations, and ultimately visible carcinomas did appear and were confirmed by biopsy. Most of the cases of carcinoma were proven rather promptly, 15 within 3 months of a cytologic diagnosis of carcinoma and 19 in the first year (Table 1). These were primarily men who had cytologic evidence of carcinoma on initial examination, and their tumors were already well developed and easily found cystoscopically.

Table 1. *Duration from first positive or suspicious cytologic finding of malignant cells to histologically proven bladder carcinoma following para-aminodiphenyl exposure*

Time	Number of cases
0–3 months	15
3–6 months	1
6–12 months	3
1–2 years	6
2–3 years	6
3–4 years	3
4–5 years	3
5–7 years	3
8, 9, 12 years	1 each

But the most interesting and most informative were the other 24 men who had had suspicious or positive cytologic findings of cancer for over a year before there was histologic confirmation on biopsy. In our initial reviews, we were surprised to find some men with recognizable cancer cells in the urine for as long as 5 or 6 years before manifest carcinoma could be confirmed by biopsy. As time has gone by, even longer periods of cytologically evident, but clinically latent carcinoma have been found: in six men for longer than 5 years, one man for 8 years, another 9 years, and a third for 12 years.

Carcinoma in situ

We felt certain that the cancer cells found in the urinary sediment during this time were coming from the urinary tract epithelium. It was our presumption that they were coming from areas of carcinoma *in situ*, that is that the normal epithelium was replaced by carcinomatous epithelial cells, and that this could not be visualized cystoscopically because there was no tumefaction and no invasive carcinoma. With this in mind, multiple biopsies were taken from different sites in the mucosa, and some of these did reveal areas of epithelium comprised of cells with enlarged, hyperchromatic nuclei, quite identical with the cancer cells we were finding in the urinary sediment (Fig. 5). The epithelium may be disorderly but there is no tumor formation and there is no invasive carcinoma. Histologically, and by definition, this is carcinoma *in situ*.

Clinical significance of carcinoma in situ. The real significance of carcinoma *in situ* depends on how it behaves clinically. Does this lesion actually progress to invasive carcinoma, and if so how long a time is required? We can answer the first question in the affirmative. So far, we have not seen biopsy proven carcinoma *in situ* of bladder regress. It will persist, or recur if removed, and given enough time most of the cases we have followed do progress to invasion.

The time intervals required for progression from *in situ* to invasive carcinoma can be determined for the industrial workers who had proven carcinoma *in situ* of the bladder, and then were followed until they developed invasive cancer. Most were treated by local measures, biopsies and fulguration. Of 10 men for whom we have this data, 8 required more than 1 year for progression, 6 required more than 3 years and 3 required over 6 years before carcinoma *in situ* progressed to invasion (Table 2).

Table 2. *Time required for progression from* in situ *to invasive carcinoma in workers exposed to para-aminodiphenyl*

Time	Number of cases
5, 6 months	2
12 months	1
2½ years	1
3 years	2
4½ years	1
6–6½ years	3

A very similar latent period is required for progression of spontaneous carcinoma *in situ* to invasive carcinoma in patients without exposure to carcinogens (Fig. 6) [7].

Symptoms. Patients with carcinoma *in situ* rarely have gross hematuria, the classical symptom of invasive carcinoma. Approximately one-fourth of the patients studied by us had no symptoms at all. When symptoms were present, they usually suggested cystitis—frequency, urgency and nocturia (Fig. 7) [7]. Because of the non-specific nature of symptoms, when they are present, carcinoma *in situ* is seldom suspected and almost never diagnosed clinically.

Gross appearance. The bladder mucosa with carcinoma *in situ* may appear perfectly normal grossly and cystoscopically. Frequently, however, it is slightly reddened and edematous, perhaps with a cobblestone or granular texture. This is due not to changes in the epithelium itself, but to congestion, edema and mild

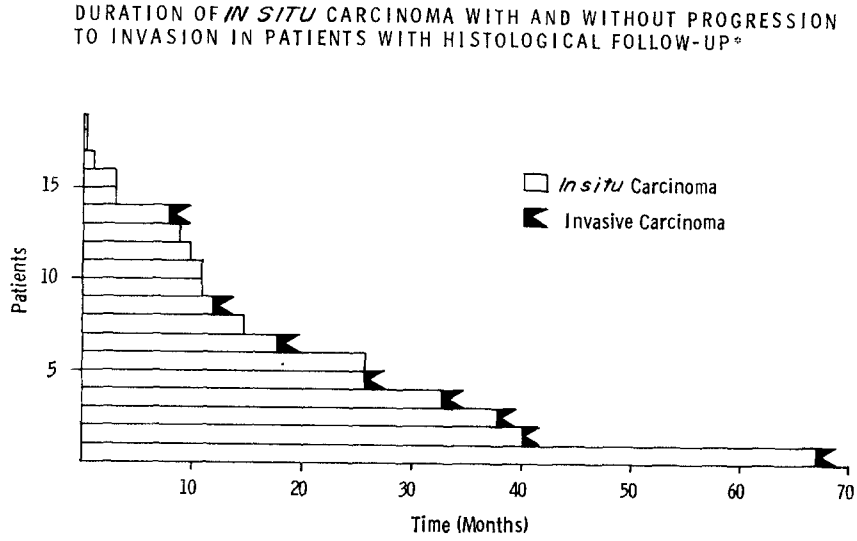


Fig. 6. Latent period required for progression of spontaneous carcinoma in situ *to invasion ranged from* 8 *months to nearly* 6 *years* [1].

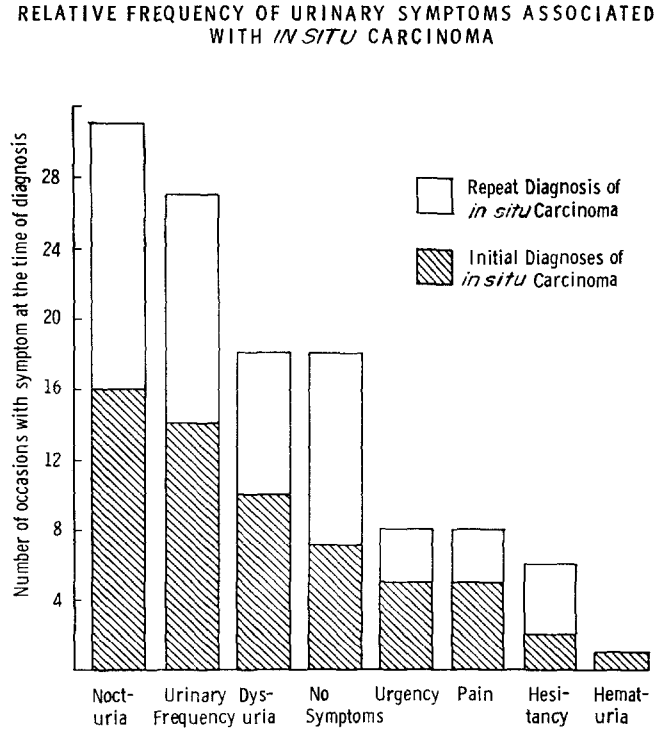


Fig. 7. Symptoms of patients with spontaneous carcinoma in situ [4].

inflammation that is often present in the stroma underlying areas of carcinoma *in situ*. Sometimes these inflammatory appearing areas of mucosa are localized, but usually they are difficult to distinguish from banal cystitis. When there are no mucosal abnormalities present in a patient suspected of carcinoma *in situ*, tetracycline u.v. fluorescence occasionally has been helpful in finding areas of abnormal mucosa to biopsy [8], but equally often it has been unreliable [9]. Our present feeling is that multiple biopsies should be taken from the base of the bladder and peri-ureteral areas when cytology suggests carcinoma *in situ* even though the mucosa appears normal, and that all areas of inflammation or other abnormality should be biopsied.

Cytology of carcinoma in situ. Most cases of carcinoma *in situ* are uncovered by cytologic examination of the urinary sediment. At Memorial Hospital, cytology is a routine part of the urologic examination, and it has been a routine part of our study of the industrial workers exposed to para-aminobiphenyl. The cells that are shed from carcinoma *in situ* show the nuclear abnormalities of cancer, but they are still fairly uniform (Fig. 8). They tend to appear singly and are usually only slightly larger than normal benign exfoliated transitional epithelial cells (Fig. 1). They do have disproportionately enlarged, hyperchromatic and irregular nuclei, there are very few highly abnormal or bizarre cancer cells present, and the background is fairly clean, showing little evidence of inflammation and usually few red blood cells [10]. This is in contrast to the very bizarre, enlarged, irregular and variable cancer cells that characterize invasive carcinoma (Fig. 2).

Also present in cytologic preparations from patients with carcinoma *in situ* are cells showing lesser degrees of nuclear abnormality. While it is not possible to do more than speculate at this point, it appears likely that these cells come from areas of borderline epithelial atypia that frequently accompany carcinoma *in situ* [9, 11–14], and may precede it in the course of carcinogenesis.

PATHOGENESIS OF BLADDER CARCINOMA

The information that we have about the development of bladder carcinoma in patients who were not exposed to known carcinogens suggests that the pathogenesis is the same for both. In both groups, for example, the tumors tend to arise at the base of the bladder and about the ureteral orifices, and to be multiple over a period of time. A recent review of the histologic patterns of invasive bladder carcinomas observed in the industrial workers showed them to be the same as spontaneous carcinomas. About three-fourths of the men had had invasive papillary carcinomas, half had had non-papillary invasive carcinomas, two of twenty-four had keratinizing squamous carcinomas and three of twenty-four had areas of adenocarcinoma associated with other patterns [15]. The pattern of growth and metastases in those industrial workers who died of their disease was the same as with spontaneous carcinoma of the bladder.

For both spontaneous and carcinogen-induced carcinomas there is evidence of a step-like progression of development that extends over a period of many years. In this progression, we can surely recognize a stage prior to invasive cancer that is identified as carcinoma *in situ*. It is occult clinically but can be discovered by cytologic examination of the urine. Interestingly, cytologic abnormalities are less marked in carcinoma *in situ* than in invasive carcinoma, consistent with the view that there is progression from one to the other.

It is also known that carcinoma *in situ* is not the first event in the development of bladder cancer. Some men exposed to para-aminobiphenyl appeared normal cytologically, clinically and cystoscopically when first examined, yet later developed bladder cancer even though the chemical carcinogen had been withdrawn long before. In some cases this period was as long as 8 years [15]. The first abnormalities that were then seen in cytologic specimens were not obvious cancer cells, but epithelial atypias that probably correspond to the borderline epithelial abnormalities associated with carcinoma *in situ*.

We presume that there is some initiating stimulus for all bladder cancers, whether it is known as in the case of the industrial workers exposed to para-aminobiphenyl, or unknown as in patients with spontaneous carcinoma. The first event must be a biochemical or biophysical abnormality at subcellular level in the epithelium. There is then a latent period of probably several years duration during which the stimulus may or may not continue to act. The patient is clinically well. Cystoscopically, the bladder is normal. Cytologically and on biopsy, the bladder mucosa and epithelium is normal.

The next stage is characterized by subtle changes in the epithelium that we call "borderline". This is a dyskaryosis, that is a change in the cell nuclei which are darker staining, somewhat enlarged and have a coarser than normal chromatin pattern.

Following that is the stage of carcinoma *in situ*, still clinically occult though now the patient may hve symptoms of cystitis and the bladder may look edematous or reddened on cystoscopy. At this stage, there are well-defined and characteristic cytologic abnormalities that can be identified on examination of the urine and confirmed on biopsy. At this stage, the disease is probably irreversible and will go on to invasive carcinoma, though there is histologic evidence of an inflammatory reaction that suggests host resistance.

The final stage in our scheme is overt invasive carcinoma. We know, again from our industrial workers, that the total course of events may be from 10–20 yr, and that each of these stages can be from months to years in duration. All our evidence from the hospital population that has not been exposed to known carcinogens suggests that this holds also for the so-called spontaneous carcinomas.

The proposed outline of carcinogenesis in the bladder can be summarized as follows:

INITIATING STIMULUS
↓
SUBCELLULAR BIOCHEMICAL OR BIOPHYSICAL ABNORMALITY
↓
"BORDERLINE" EPITHELIAL ABNORMALITIES
↓
CARCINOMA *IN SITU*
(Clinically Occult—Cytologically Manifest)
↓
OVERT INVASIVE CARCINOMA

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The Induction of Immunoprotection by Mouse Ascites Tumor Cells Attenuated in Tissue Culture*

J. F. MORGAN and C. P. ENG†

Departments of Biochemistry and Cancer Research, University of Saskatchewan, Saskatoon, Canada

Abstract—*The loss of transplantability and high degree of immunoprotection shown by 6C3HED-ascites tumor cells in tissue culture have been confirmed with the TA3 ascites tumor. The induction of immunoprotection against solid as well as ascites tumor formation has been demonstrated with 6C3HED non-tumorigenic tissue culture cells. Treatment of virulent 6C3HED ascites cells by chemical or physical agents destroyed completely their immunizing capacity. Similar treatment of non-tumorigenic tissue culture cells reduced but did not destroy their immunizing capacity.*

INTRODUCTION

THE PIONEER experiments of Gey in 1941 [1] and of Earle in 1943 [2] demonstrated that normal rat and mouse tissues cultivated *in vitro* for prolonged periods underwent a progressive series of changes resulting in the development of some malignant characteristics. This observation of a trend towards malignancy in cell cultures derived from normal tissues has been thoroughly proved by many other investigators and has become an accepted concept of tissue culture research.

Long-term tissue cultures derived from tumors have also been shown to lose their transplantability. This effect was first demonstrated by De Bruyn and Gey [3] in 1952 with mouse lymphoma cells and has subsequently been confirmed by Hsu and Klatt [4] with rat hepatoma cells, by Foley and Drolet [5] with mouse Sarcoma 180 cells, and by Moore *et al.* [6] with Ehrlich ascites cells of the mouse. Recently, Harris *et al.* [7], and Watkins and Chen [8] have hybridized Ehrlich ascites cells with mouse fibroblasts or with virus-transformed hamster cells and have shown loss of transplantability associated with retention of immunoprotective capacity.

In the authors' laboratory, a series of tissue culture lines established from the Ehrlich, TA_3, Ehrlich-Lettré and 6C3HED mouse ascites tumors were found to have lost their transplantability after from 1 to 5 years of propagation *in vitro* [9]. The establishment of new and still tumorigenic tissue culture lines from these mouse tumors has made it possible to develop a three-component model system for the study of this phenomenon, based on the tumors *in vivo* together with their derived tumorigenic and non-tumorigenic cell cultures.

The present paper reports data on the immunoprotective capacity of these non-tumorigenic cell cultures and suggests that attenuation of tumor cells through tissue culture might provide a basis for protection against some types of animal tumors.

MATERIAL AND METHODS

Tumors

Hypotetraploid lines of the 6C3HED, Ehrlich, TA_3, and Ehrlich-Lettré ascites tumors were maintained by weekly serial passage with the intraperitoneal inoculation of $2–4 \times 10^6$ cells per mouse. The first 3 tumor lines were obtained

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†Present address: Bionetics Research Laboratories, Inc., 5510 Nicholson Lane, Kensington, Md. 20795, U.S.A.

from Dr. G. A. LePage, Stanford Research Institute, Menlo Park, California, and the Ehrlich-Lettré tumor line from Dr. M. J. Fraser, University of Manitoba, Winnipeg, Canada. All 4 lines have been carried for many years in the authors' laboratory. These tumors provided cells for the establishment of tissue culture lines and also virulent cells to provide challenge doses in immunoprotection experiments.

All studies with the 6C3HED tumor were conducted in male C3H mice, weighing 20 to 25 g, either purchased from the Jackson Laboratory, Bar Harbor, Maine or bred at the University of Saskatchewan from Bar Harbor stock. With the other 3 tumors, male Swiss mice weighing 23 to 28 g, from Connaught Medical Research Laboratories, University of Toronto, Canada, were used. These mice were bred under barrier-sustained conditions at the University of Saskatchewan. All mice under experiment were supplied with food and water *ad libitum*.

Mice were inoculated either intraperitoneally or subcutaneously with varying doses of cells established by the Coulter Counter. They were then observed for 90 days and the number dying from tumor development was recorded. At the end of each experiment, any surviving mice were killed and examined for solid tumor formation either in the peritoneal cavity or at the site of inoculation.

Tissue culture

Cell lines were established as monolayer cultures in Medium 199 [10], supplemented with 5% calf serum [11], by procedures described previously [12]. All ascites cell cultures were treated every 3 months with 500 μg/ml kanamycin (supplied by Bristol Laboratories, Inc., Syracuse, N.Y.) to eliminate any chance of mycoplasma contamination. After prolonged cultivation *in vitro*, the ascites cell lines failed to produce tumors on back transplantation and were used for immunization studies. For passage, the cells were scraped, aspirated and then transferred to new culture vessels in a 1:2 or 1:3 ratio. This method produced maximum monolayer cultures in 6–8 days. Cell number was determined with a hemocytometer, and cell viability was measured by the dye exclusion test, using erythrosin B. The viability of the non-tumorigenic tissue culture cells ranged between 72 and 81%, while that of the virulent tumor cells taken directly from the mouse always exceeded 96%.

Immunization

Non-tumorigenic tissue culture cells were harvested, washed 3 times in 10 volumes of Hanks' balanced salt solution (HBSS) [13], and resuspended to the desired concentration in HBSS. Each mouse then received intraperitoneally $5–8 \times 10^6$ cells in 0·5 ml suspension. The injections were administered once weekly for 3 weeks. One week after the final injection the mice were challenged either intraperitoneally or subcutaneously with varying numbers of virulent tumor cells freshly drawn from a mouse. At the same time, equal numbers of normal non-immunized mice were given injections of viable tumor cells from the same cell suspension. All mice were then kept for a 90-day observation period.

Washed, non-tumorigenic 6C3HED or TA3 tissue culture cells were suspended in HBSS to a concentration of $8·0 \times 10^6$ cells per ml and were then treated for 60 sec in an MSE sonic oscillator. Microscopic observation indicated that the cells were completely disrupted after this treatment. This material was then used for immunization purposes as described previously. Similar experiments were conducted with virulent 6C3HED or TA3 cells freshly harvested from mouse tumors.

Virulent ascites tumor cells and their derived non-tumorigenic tissue culture lines were incubated with 10^{-3} M sodium iodoacetate at 37°C for 1 hr, according to the procedure of Apffel *et al.* [14]. These devitalized cells were then used for immunization experiments.

Tumor cells and their derived non-tumorigenic tissue cultures received X-irradiation in a Picker Corporation instrument at 200 kVp and 18 mA using a 0·25 N filter. Both types of cells received a total of 4000 rads at an exposure rate of 50 rads/min with a focal distance of 12·1 cm. The dose rate was calibrated with a Baldwin-Farmer dosemeter.

Specificity of immunity

Groups of Swiss mice were immunized by weekly intraperitoneal injection for 3 weeks with either spleen cells from C3H mice or with 6C3HED non-tumorigenic tissue culture cells. Subsequently, both groups of mice were challenged with $1·0 \times 10^4$ virulent cells of the 6C3HED, TA3, Ehrlich, and Ehrlich-Lettré ascites tumors. The mice were then held for 90 days and tumor formation was recorded [15].

Chromosome analyses of the ascites tumor cells and of their derived tumorigenic and non-tumorigenic tissue cultures were performed as described previously [16]. Mice were subjected to total body X-irradiation in a Picker therapy machine at 200 kVp and 20 mA.

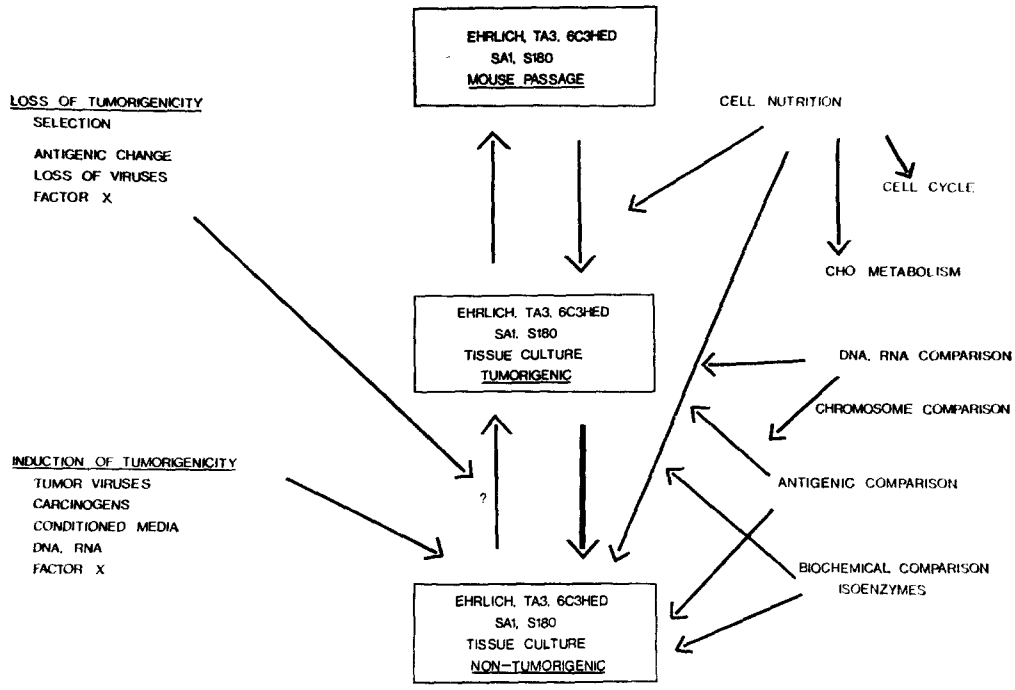

Fig. 1. *General research program for study of the attenuation phenomenon. Central portion of the diagram illustrates the three-component tumour model system developed.*

RESULTS

Attenuation phenomenon

The research program that has been developed to study the phenomenon of attenuation is shown diagrammatically in Fig. 1. The essential part of the system is shown in the central portion of the diagram. It consists of a spectrum of fully virulent ascites tumors maintained by weekly serial passage in mice. From these tumors, fully transplantable tissue culture lines are established which become non-transplantable after long-term cultivation. The system is maintained by the frequent establishment of new tumorigenic tissue culture lines to replace those that have become non-transplantable.

The most important features of the tumorigenic and non-tumorigenic ascites cells are summarized in Table 1. The great difference between the tumorigenic and non-tumorigenic cell cultures is shown in their relative transplantability. The tumorigenic culture normally produced 100% tumors with the injection of 10^2 cells, while the non-tumorigenic cell cultures failed to produce any tumors with the intraperitoneal injection of as many as 10^8 cells. Similar results were obtained whether normal mice were used or mice which had been subjected to total body X-irradiation to suppress their immune mechanisms.

Table 1. *Important characteristics of tumorigenic and non-tumorigenic ascites cells*

In vivo	1.	Grow very slowly in tissue culture
	2.	75–78 chromosomes
	3.	100% tumours produced by 10^2 cells
In vitro		
Tumorigenic		
	1.	Grow rapidly in tissue culture
	2.	Average 123 chromosomes, with 1 metacentric
	3.	100% tumors produced by 10^2 cells
Non-tumorigenic		
	1.	Grow rapidly in tissue culture
	2.	Average 117 chromosomes, with 3 or 4 metacentrics
	3.	O tumors with as many as 10^8 cells

Immunoprotection

The extent of the tumor-specific immunity induced by the non-tumorigenic tissue culture cells was studied by immunizing groups of mice with 6C3HED or TA3 cells and challenging with the homologous ascites cells, as summarized in Tables 2 and 3. A very high degree of immunoprotection is evident, with 95% protection against challenge of 10^6 6C3HED cells and 70% protection at this dose with TA3 cells. When 10^7 6C3HED cells were used for the challenge dose, the immunoprotection was decreased to 60%.

*Table 2. Immunoprotection induced by 6C3HED non-tumorigenic tissue culture cells against challenge with virulent 6C3HED ascites cells at different dosages**

Challenge dose (no. of cells inoculated per mouse)	Normal mice	Immunized mice	
	Survivors†	Survivors†	Protection (%)
10^3	0/20	10/10	100
10^4	0/20	20/20	100
10^5	0/20	20/20	100
10^6	0/20	19/20	95
10^7	0/20	12/20	60

*Immunized mice received intraperitoneal injections of 5–8 × 10^6 non-tumorigenic cells once a week for 3 weeks and were challenged with virulent cells 1 week later.

†Expressed as number of mice surviving for 90 days/number of mice challenged.

*Table 3. Immunoprotection induced by TA3 non-tumorigenic tissue culture cells against challenge with virulent TA3 ascites cells at different dosages**

Challenge dose (no. of cells inoculated per mouse)	Normal mice	Immunized mice	
	Survivors†	Survivors†	Protection (%)
10^2	0/20	20/20	100
10^3	0/20	20/20	100
10^4	0/20	20/20	100
10^5	0/20	20/20	100
10^6	0/20	14/20	70

*Immunized mice received intraperitoneal injections of 5–8 × 10^6 non-tumorigenic cells once a week for 3 weeks and were challenged with virulent cells 1 week later.

†Expressed as number of mice surviving for 60 days/number of mice challenged.

Two mice which received intraperitoneal injections of 10^6 virulent TA3 ascites cells are shown in Fig. 2. The normal control mouse (right) has died from an ascites tumor while the pre-immunized mouse (left) shows no sign whatever of tumor development. Similar results were obtained with 6C3HED cells.

Effect of chemical and physical treatments

Previous studies from this laboratory [15] had established that maximum efficiency of immunization was obtained when more than 10^5 non-tumorigenic cells were administered at each injection and that two or more immunizing doses were most effective. It was considered of interest to determine the effect of chemical and physical treatment of both tumorigenic and non-tumorigenic cells upon their immunizing capacity, as summarized in Table 4. It is evident that the physical and chemical treatments used have completely devitalized the virulent ascites cells since no tumors were elicited by injection of the treated cells. These treatments, however, have also destroyed the immunizing capacity of the cells since no protection whatever is afforded against subsequent challenge with virulent cells. In contrast, the attenuated tissue culture cells still retain a high proportion of their immunizing capacity after the chemical and physical treatments. X-irradiation and iodoacetate appear to be less damaging in their effects than are lyophilization, sonication or freezing and thawing.

Passive immunity and cross-protection

Our previous studies [15] have shown that the immunoprotection induced by non-tumorigenic 6C3HED tissue culture cells can be demonstrated by both the serum and the spleen

*Table 4. Effect of chemical and physical treatments on immunoprotective capacity of virulent and attenuated 6C3HED cells**

Treatment of cells before immunization	Treatment controls (virulent cells) Survivors	Immunization with		
		Virulent cells Survivors	Attenuated cells Survivors	Protection (%)
No treatment	0/20	—	20/20	100
Iodoacetate	20/20	0/20	15/20	75
Lyophilization	20/20	0/20	11/20	55
Freezing and thawing	20/20	0/20	13/20	65
Sonication	20/20	0/20	12/20	60
X-irradiation	20/20	0/20	16/20	80

*Results are expressed as number of mice surviving after 60 days/number of mice inoculated. Immunizations were carried out with 5 × 10^6 cells intraperitoneally once a week for 3 weeks, followed by challenge with 10^5 untreated virulent cells 1 week later.

Fig. 2. *Two mice inoculated intraperitoneally with* 10^6 *virulent TA*3 *ascites cells. Mouse on left had been pre-immunized with non-tumorigenic TA*3 *tissue culture cells. Non-immunized control mouse is on right.*

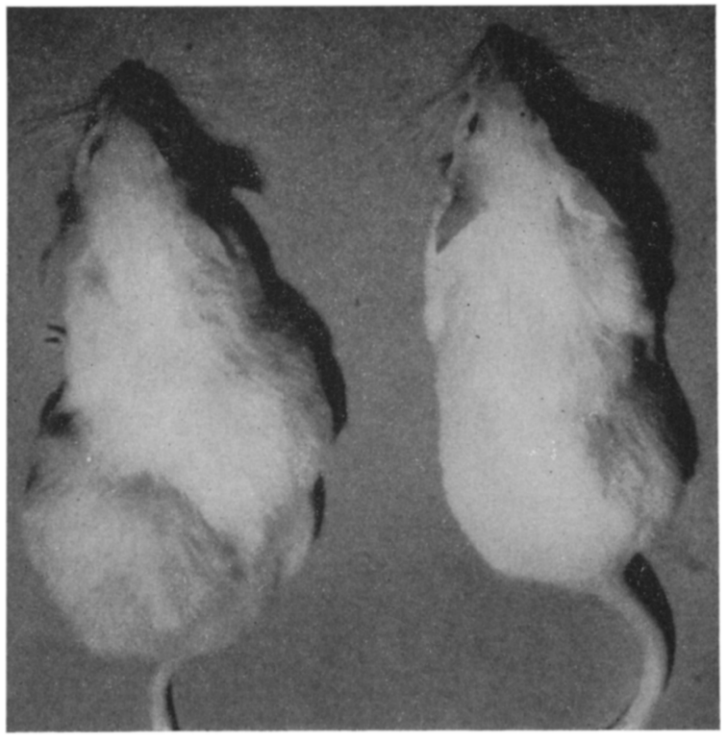

Fig. 3. *Two mice inoculated subcutaneously in left thigh with* 10^6 *virulent TA*3 *ascites cells. Mouse on right had been pre-immunized with non-tumorigenic TA*3 *tissue culture cells. Non-immunized control mouse is on left and shows development of large solid tumour.*

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cells of the immunized mice. Protection rates of 73% and 70% were obtained, respectively, against challenge with 10^4 virulent 6C3HED cells.

Mice immunized with 6C3HED non-tumorigenic tissue culture cells showed a considerable degree of crossprotection when challenged with virulent TA3, Ehrlich or Ehrlich-Lettré cells [15]. The degree of crossprotection achieved was 40% for TA3 cells, 60% for Ehrlich-Lettré cells, and 70% for Ehrlich cells, while 100% protection was obtained against the homologous 6C3HED cells. The high degree of crossprotection obtained suggests that some common antigens have been developed during the long-term cultivation *in vitro*.

Immunoprotection against solid tumors

Ascites cells inoculated subcutaneously rather than intraperitoneally produce large solid tumors at the site of injection. Since the non-tumorigenic tissue culture lines had been shown to induce a strong immunoprotection against challenge with virulent cells administered intraperitoneally, it was considered of interest to determine whether such immunity would extend to the solid tumor system. Accordingly, groups of C3H mice were immunized with non-tumorigenic 6C3HED tissue culture cells and were then challenged with graded doses of virulent 6C3HED cells injected subcutaneously in the dorsal region of the right thigh. The results summarized in Table 5 show that a high degree of immunoprotection, totaling 90% at a challenge dose of 10^7 cells, has been attained. This degree of immunoprotection is somewhat higher than that recorded previously for intraperitoneal challenge in the same cell system [15].

Table 5. *Immunoprotection induced against solid tumor formation with attenuated 6C3HED cells**

Challenge dose	Normal mice	Immunized mice	Protection (%)
10^3	10/20	0/20	100
10^5	19/20	0/20	100
10^7	20/20	2/20	90

*Results expressed as number of mice developing solid tumours/number of mice challenged with virulent 6C3HED cells subcutaneously.

Two mice which received subcutaneous injections of 10^6 virulent TA3 cells in the left thigh are shown in Fig. 3. The normal control mouse (left) has developed a very large solid tumor while the pre-immunized mouse (right) shows no sign whatever of tumor development.

DISCUSSION

The previous observation [9, 15] that 6C3HED ascites tumor cells become non-tumorigenic after prolonged cultivation *in vitro*, but retain a high immunoprotective capacity has now been confirmed with TA3 ascites tumor cells. With both cell lines nearly complete protection has been achieved against a challenge dose of 10^6 fully virulent ascites cells. Extension of these studies has shown that immunoprotection can be achieved against solid tumor as well as ascites tumor formation at a similar high challenge dose of virulent cells. This observation is in accord with our previous report [15] that either spleen cells or sera from immunized mice could neutralize the transplantability of virulent tumor cells *in vivo*. It would appear that the immunoprotection elicited by non-tumorigenic tissue culture cells is a whole body immunity response.

When virulent ascites cells were devitalized by chemical or physical treatments they failed to induce immunoprotection in mice. Following similar treatments, the non-tumorigenic tissue culture cells still retained most of their antigenic capacity, although such treatments as lyophilization and sonication reduced this capacity to a considerable extent. It would appear from these results that untreated nontumorigenic cells are far more effective as immunizing agents than are devitalized virulent ascites cells.

The concept of the loss of transplantability by tumor cells during long-term tissue culture is not a new one since the idea of attenuation was expressed clearly by Hsu and Klatt [4] in 1959 and by Sanford *et al.* [17] in 1955. The present studies appear to be unusual in the complete loss of transplantability obtained, coupled with an extremely high antigenic capacity. The fact that a moderate degree of cross-protection against other ascites tumors can be developed [15] and that the immunity can be transferred by spleen cells or serum suggest that the phenomenon of attenuation deserves intensive study.

The mechanism of the loss of transplantability has not yet been established. Selection of non-tumorigenic cells from a heterogeneous population, some form of regression from the malignant state, or some form of cell hybridization appear to be obvious possibilities. The attenuation process appears to occur spontaneously and on a different time basis for each ascites tumor line studied. If the mechanism of attenuation could be elucidated it might become possible to expedite the process and control it experimentally, steps that would be crucial in the development of attenuated cells for therapeutic purposes.

Acknowledgements—The financial support of the Medical Research Council of Canada is gratefully acknowledged. We wish to express our appreciation to Mrs. M. D. Heuchert for skilled technical assistance.

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Phorbol Esters as a Tool in Cell Research?*

R. SÜSS, G. KREIBICH and V. KINZEL
Deutsches Krebsforschungszentrum Heidelberg, 69 Heidelberg, Berlinerstr. 29, Germany

Abstract—*The potent tumour-promoting phorbol ester TPA obtained from croton oil leads,* in vitro, *in very low concentrations of HeLa-cells* ($10^{-8}M$) *to specific effects which are not caused by analogous biologically inactive phorbol esters. Firstly, the time-dependent depression of thymidine incorporation suggests a block before S-phase in the cell cycle; secondly, the excessive stimulation of choline incorporation suggests greatly increased membrane metabolism. TPA stimulates choline corporation into 3T3-cells without depressing thymidine incorporation. Cell numbers in TPA-treated 3T3 cultures are higher than in controls.*

PHORBOL esters are of interest in initiation-promotion mechanisms of carcinogenesis[1–3]. A mouse is treated with a subthreshold dose of a carcinogen such as DMBA which alone does not produce tumours, and then painted repeatedly with diluted solutions of croton oil, which cause papillomas to develop. This croton oil treatment has been called tumour promotion and accordingly croton oil is a tumour promoter.

However, croton oil does more than promote latent tumour cells into visible papillomas: a single application of croton oil on the back skin of a mouse starts an excessive cell proliferation. But this is not the only action of croton oil. In addition to tumour promotion and hyperplasia, inflammation with hyperaemia, oedema and vascular changes are produced [4], immunosuppression has been detected [5], and last but not least, croton oil is mildly carcinogenic [6].

This rather disturbing multiplicity of effects was the reason for initiating research to isolate pure tumour promoters from croton oil which led to the discovery of biologically active phorbol esters. The most important of these compounds is Tetradecanoyl-phorbol acetate (TPA) [7, 8]: a complicated ring system carries at the positions 12 and 13 two fatty acids, one with 14 C-atoms and one with 2. The knowledge of the chemical structure and the ability to synthesize artificial tumour promoters encouraged the hope of finding criteria by which a tumour promoter is defined. However, even if one knows precisely the chemical structure of a tumour promoter, one still does not know the mechanism of action of a tumour promoter. The effects of TPA are practically as versatile as croton oil. This makes it a problem to plan clearcut biochemical experiments. Furthermore, mouse skin is a tough tissue and difficult to handle by usual biochemical techniques.

We decided to turn away from tumour promotion, hyperplasia, inflammation and even mouse skin and to ask the simple question: how do phorbol esters act in cell culture, and how do they act on conventional cell lines? Although L-cells, HeLa-cells, 3T3-cells are not epidermal cells, if phorbol esters interfere with some basic molecular mechanisms, this interference might show up in cell types other than epidermal cells.

The study of phorbol esters in cell culture was based at first on simple biochemical parameters. The cells were incubated with phorbol esters, they were pulsed with radioactive precursors and the incorporation was stopped with SDS, after which the cells lysed. An aliquot of the

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lysate was pipetted onto filter paper discs and the radioactivity counted according to Mans and Novelli [9].

In the first phase of our work, the actions of three compounds were tested:

(1) TPA (see above).

(2) A synthetic ester, carrying at the positions 12 and 13 a fatty acid with 10 C atoms, which is called phorbol-di-decanoate, abbreviated as PDD.

(3) A biologically inactive phorbol ester, called 4-PDD. This compound is stereoisomeric to PDD with the only difference at position 4: the hydroxyl group at this position either stands upwards or backwards.

Experiments with L-cells proved to be disappointing. L-cells were incubated for 6 hr with TPA and pulsed with tritiated thymidine (^{3}H TdR) for 1 hr. Only at very high concentrations of TPA (10^{-4}M) was ^{3}H TdR incorporation inhibited, the cells died and lysed. Treatment of L-cells with a detergent or inactive phorbol-ester gave similar results.

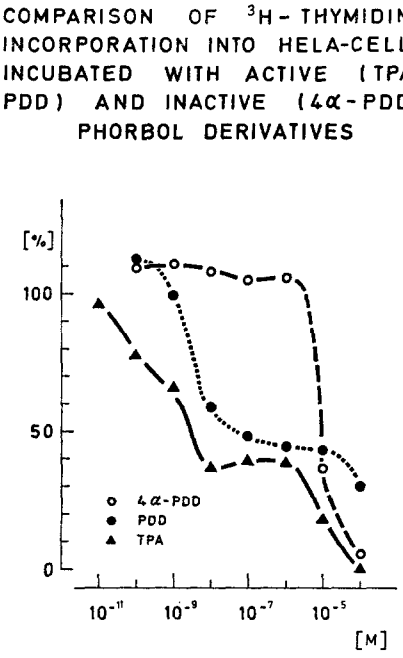

Fig. 1. Thymidine incorporation into HeLa-cells incubated with croton oil factor TPA, with PDD and 4α-PDD for 6 hr ("thymidine terrace" of biologically active phorbol esters).

Figure 1 shows ^{3}H-TdR incorporation into HeLa-cells, treated for 6 hr with TPA; the concentrations are plotted on the abscissa. HeLa-cells react in a more sensitive way than L-cells: at low concentrations—starting from 10^{-8} M—^{3}H-TdR incorporation is reduced by more than 50%. This inhibition then remains constant even if the concentration of TPA is increased one hundred-fold to 10^{-6} M.

At high concentrations of TPA, ^{3}H-TdR incorporation is reduced to zero. At this concentration range the cells are lysed, though they remain intact in the plateau phase.

Therefore one can distinguish between two different effects of TPA on ^{3}H-TdR incorporation into HeLa-cells. (1) Simple lysis, dead cells no longer incorporate thymidine; (2) a non-lytic blockage of DNA synthesis at lower concentrations. These concentrations are indeed rather low: 10^{-8} M corresponds to not more than 0·006 μg/ml.

It is possible to differentiate between lytic and non-lytic action other than by microscopy.

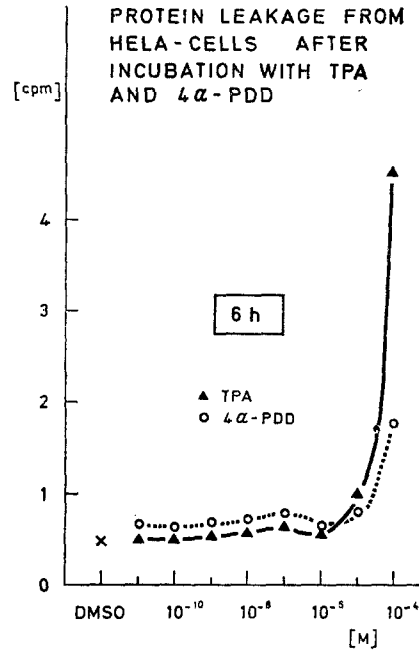

Fig. 2. Protein leakage from prelabelled HeLa-cells after incubation with TPA and 4α-PDD for 6 hr.

Figure 2 shows HeLa-cells which had been pre-labelled with radioactive leucine before they were incubated with TPA for 6 hr. Then the radioactivity passing into the medium was determined and it can be seen that only at 10^{-5} and 10^{-4} M are significant amounts of radioactivity lost from the cells. Inactive 4α-PDD gives rather similar results. We compared ^{3}H-TdR incorporation in the presence of the three standard phorbol esters (Fig. 1). Both active compounds TPA and PDD give the plateau pattern, but biologically inactive 4α-PDD does not. This suggests that HeLa-cells can differentiate between active and inactive phorbol esters, whereas L-cells cannot.

One may speculate that HeLa-cells are more sensitive because they are epithelial cells like the presumptive target cells in mouse epidermis. On the other hand, L-cells do not appear to be an appropriate target for phorbol esters. This

speculation is supported by the observation that other epithelial cells such as liver cells or kidney cells reacted like HeLa-cells. However, there is a more detailed explanation: HeLa-cells do not metabolize TPA or PDD, and neither do L-cells. If one assumes that it is the phorbol ester itself that is biologically active, then it is easily understood that only HeLa-cells are sensitive because only in these cells would unchanged phorbol esters be available.

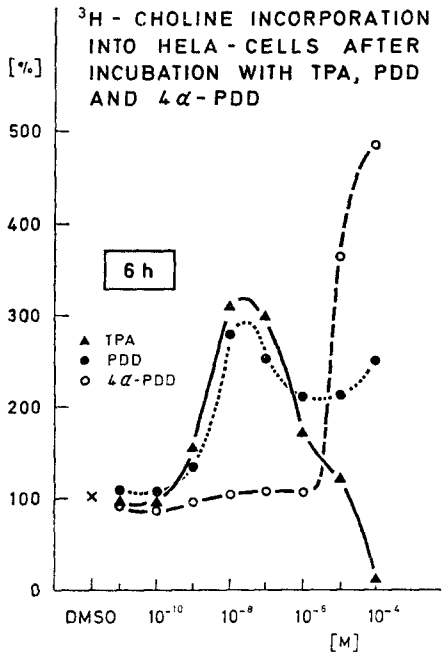

Fig. 3. Choline incorporation into HeLa-cells after incubation with TPA, PDD and 4α-PDD for 6 hr.

In contrast to ^{3}H-TdR uptake, choline incorporation is dramatically stimulated by TPA. Choline is incorporated into lecithin, lecithin into lipids and lipids can be built into membranes. Therefore the increased incorporation of choline could reflect a stimulation of membrane synthesis. This dramatic stimulation fails to appear if protein synthesis is blocked. In the presence of actidione, most of the stimulation is prevented. The choline stimulation is restricted to active phorbol ester (Fig. 3): TPA and PDD stimulate, but 4α-PDD does not, at least not at low concentrations.

Figure 4 shows the ^{3}H-TdR and choline data plotted together: choline stimulation occurs at the same low concentrations at which the thymidine uptake plateau is observed. It can be questioned whether this pattern is typical for phorbol esters.

In the second phase of our work many phorbol derivatives provided by Dr. Hecker were tested. We speeded up the Mans–Novelli pro-

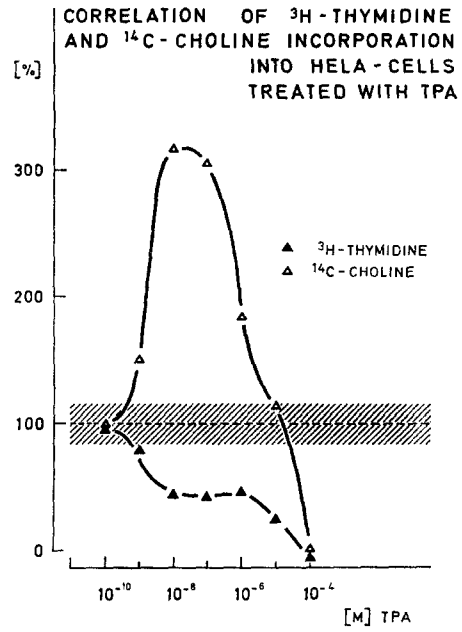

Fig. 4. Correlation of stimulated choline and reduced thymidine incorporation into HeLa-cells treated for 6 hr with TPA.

cedure with the filter paper discs using a pin-up board (Fig. 6a). After being processed, the discs are released into counting vials, simply by pressing the board down (Fig. 6b). Switching to plastic culture trays, commercially available from Linbro Plastics, made the test procedure faster. We call these trays Single Culture Screening Trays (SST).

The whole device consisting of a SST and a protective lid is pressed together and incubated in a CO_2 incubator. The cell cultures are processed as usual; however, after fixing, extracting and drying, the base of each of the troughs is cut off with scissors and directly dropped into counting vials.

In Table 1 some of the tested compounds are listed with a plus sign standing for inflammatory or co-carcinogenic activity on the mouse skin or thymidine depression and choline stimulation in HeLa-cells. It can be seen that active compounds are positive *in vivo* as well as *in vitro*, whereas inactive compounds are negative throughout the whole table. A good correlation between chemical structure and biological activity has been found in the series of phorbol esters with varying chain lengths. A chain length of less than four C-atoms or more than twelve C-atoms makes them inactive. This is again very well reflected in our *in vitro* system.

Table 1 includes two isomeric ethers (EPT and TPE); one of them is a tumour promoter, the other not. Here again either in all positions + or in all positions −. The formulae of these two compounds show only minimal variations:

Table 1. In vitro *and* in vivo *activities of phorbol esters*

Compound tested	Test systems			
	Mouse skin		HeLa cells	
	Irritation	Tumour-promotion	^{3}H-TdR decrease	Choline increase
TPA	+	+	+	+
PDD (Dic_{10})	+	+	+	+
4α-PDD (ISO)	−	−	−	−
P-Di-C_{10}-4, 10-diacetate	−	−	−	−
P-DiC_{10}-4-acetate	−	−	−	−
P-DiC_{10}-9-acetate	−	−	−	−
P-13-monoacetate	−	−	−	−
Phorbol	−	−	−	−
P-DiC_2	−	−	−	−
P-DiC_4	+	(+)	+	+
P-DiC_6	+	+	+	+
P-DiC_8	+	+	+	+
P-DiC_{10}	+	+	+	+
P-DiC_{14}	−	−	−	−
P-DiC_7	+	(+)	+	+
EPT	+	+	+	+
TPE	−	−	−	−

Abbreviations used: TPA = Tetradecanoyl-phorbol-acetate, PDD = Phorbol-didecanoate, P = Phorbol, P-DiC_2 = Phorbol-diacetate, P-DiC_4 = Phorbol-dibutyrate, etc., EPT = Ethyl-phorbol-tetradecanoate, TPE = Tetradecanoyl-phorbol-ethylether.

in one case the ether group is attached to position 12, in the other case to position 13. Nevertheless, a mouse skin can easily distinguish between these two ethers and the same is true for HeLa-cells.

Our biochemical reaction pattern (Fig. 4) seems, therefore, to be specific for biologically active phorbol esters. However, what does it mean, how can the thymidine depression plateau and the choline stimulation be interpreted?

When thymidine incorporation is plotted in relation to time it can be seen that immediately after the addition of TPA, thymidine incorporation is depressed in a rather linear fashion (Fig. 5). It does not matter which concentrations are used, provided they are low; 10^{-8} to 10^{-6} M gave the same result. Uridine incorporation partly follows thymidine, it is depressed but then it levels off at about 70% after 9 hr. Leucine incorporation is very little affected initially and it remains unaffected with prolonged incubation.

Choline follows a rather different pattern (Fig. 5). Whereas thymidine incorporation was depressed, choline incorporation reached a peak and returned to normal. One possible explanation could be that: if, within a cell cycle, arrest occurred just before S-phase, so that no cell

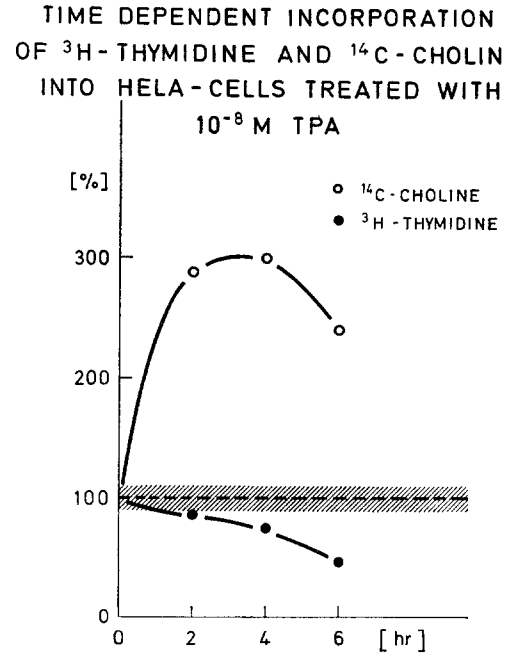

Fig. 5. Time-dependent incorporation of thymidine and choline into HeLa-cells treated with 10^{-8} *M TPA.*

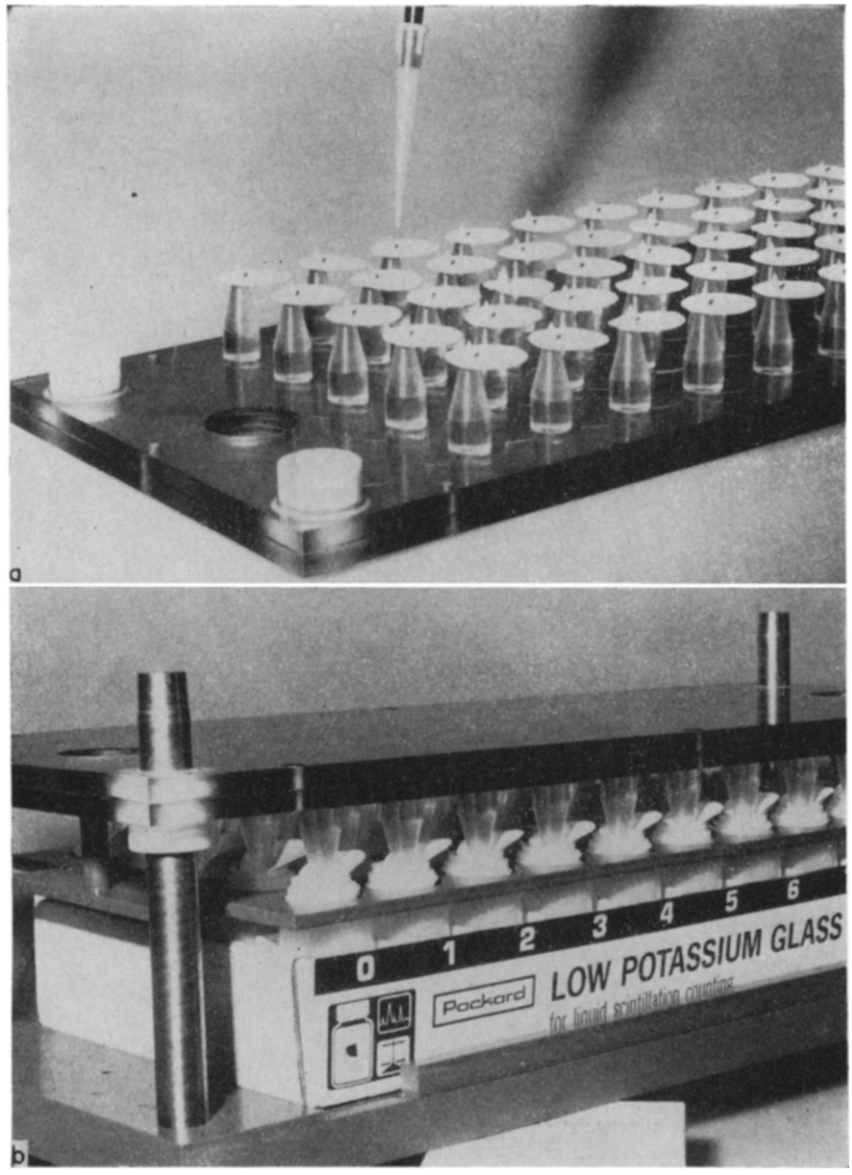

Fig. 6. *Automatic pin-up board for filter paper discs: the pin-up board (a) is loaded with the paper discs using a loading board (not shown here). After processing and drying, the discs are placed into counting vials simply by pressing the board down (b). (This machine may be obtained from Packard Instrument, Zürich.)*

(to face p. 302)

could enter DNA-synthesis, all cells just cycling through S-phase could finish their DNA replication. The number of cells would fall off rather linearly. As the last cell finished its S-phase, the thymidine incorporation would be zero. It would be expected that after 6 to 8 hr all cells would be through S-phase. In our experiments, however, it took a little longer. Obviously, the simple concept of a blockage or disturbing a trigger system is not absolutely true. However, we have some confidence that it is basically sound, as Dr. Mueller observed a similar phenomenon using synchronized HeLa-cells [10].

We conclude that TPA arrests cells before S-phase, that this block is very close to the beginning of S-phase, and that TPA does not affect DNA synthesis *per se*.

This is an oversimplification, as TPA affects mitoses directly, and DNA synthesis is not only shut off but slowed down as well, as shown by autoradiography.

Croton oil factor TPA obviously induces membrane changes which set off processes finally leading to a blockage of S-phase. TPA may release the P1 proteins that Green has recently described [11] or it may release simple cyclic AMP. It is quite fashionable to relate DNA synthesis with membranes, and not only in bacterial systems. Even in mammalian cells newly synthesized DNA has been found to be associated with nuclear envelopes [12]. Thus it may be reasonable to assume that the TPA-induced membrane changes affect DNA synthesis.

We supplemented our studies of phorbol esters activity on cell cultures with experiments using 3T3 cells. In contrast to HeLa-cells thymidine incorporation was not affected (after 6 hr), whereas stimulation of choline incorporation was even higher than in HeLa-cells. In the long run however DNA-synthesis *is* affected: cell counts revealed that TPA promotes cell proliferation; after three days an increase of some 25% was counted. Van Duuren already reported on similar observations one year ago [13].

The possible relevance of these observations to the action *in vivo* of the croton oil factor is as follows: to explain hyperplasia in the mouse we can propose at least four models, all starting with stimulating lipid metabolism which occurs *in vivo* and *in vitro* [14, 15].

(1) Leaky cells could lose growth-controlling chalones. (2) Dead cells could lead to a reparative regeneration to make up for the damage. (3) The reaction of HeLa-cells suggests another model, even if at first sight it seems contradictory that blocking of S-phase should explain increased DNA-synthesis. However, the cell cycle offers an interpretation: the longer the blockage occurs before the S-phase the more cells will be arrested and will have to wait here. Lifting the blockade would allow more cells to pass through S-phase than under normal conditions. In cell culture this opening can be done by changing the medium [16]. In fact, if the medium is changed 24 hr after TPA had been added, thymidine incorporation goes up and goes much higher than in the controls. (4) 3T3-cells provided another model because these cells can be stimulated by TPA without foregoing inhibition of DNA-synthesis.

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Cell Kinetics of African Cases of Burkitt Lymphoma

A preliminary report*

ULLA IVERSEN, OLAV HILMAR IVERSEN, AVRUM Z. BLUMING, JOHN L. ZIEGLER and SEBASTIAN KYALWASI

Uganda Cancer Institute, Lymphoma Treatment Center and Department of Pathology, Makerere Medical School, Kampala, Uganda, and Institutt for Generell og Eksperimentell Patologi, University of Oslo, Rikshospitalet, Oslo 1, *Norway*

Abstract—*Small pieces of fresh biopsies of tumour tissue from* 17 *patients with African Burkitt lymphoma and three patients with other non-Burkitt undifferentiated lymphosarcomas were transferred to tissue culture medium with tritiated thymidine and Colcemid. Specimens were fixed after* 1, 2, 3 *and* 4 *hr respectively, autoradiographed, and labelling indices determined. In two patients in stage* IV, *tritiated thymidine was given intravenously every* 6 *hr for* 2 *days. Biopsies were obtained* 1 *hr after the first injection, and then on the first and the second day, and the labelling indices were determined. In the same two patients the diameters of cutaneous tumour nodules were also measured for* 3 *days.*

The results show that in the Burkitt tumours analyzed, the mean cell "birth-rate" was 2·71% *of all cells per hr, which gives a potential doubling time of* 25·5 *hr. The actual doubling time was* 66 *hr. The growth fraction was estimated to be* 90–100% *of all cells. The rate of cell loss was calculated to be about* 70% *of the rate of cell renewal. The rate of cellular proliferation in the three lymphomas of non-Burkitt type was* 3·78% *of all cells per hr, which gives a potential doubling time of* 18·3 *hr.*

The rapid rate of cell turn-over in the Burkitt tumour may kinetically explain the rapid response to cytostatic treatment, but it can not explain why Burkitt tumours seem to be more curable than other lymphomas. Immunological mechanisms may be responsible for this.

INTRODUCTION

THE AFRICAN Lymphoma, or Burkitt tumour, is of considerable interest because of its rapid growth rate and its remarkable response to cytotoxic drugs [1]. Previous kinetic studies *in vitro* on cell lines derived from such tumours have revealed remarkably short cell-cycle times of the proliferating cells and a high rate of cell loss. It was, therefore, of interest to study the cell proliferation kinetics in small pieces of organized tumour tissue to obtain numerical values on the rate of cell proliferation.

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MATERIAL AND METHODS

We obtained fresh biopsies from 25 patients attending the Lymphoma Treatment Center, at Makerere Medical School in Kampala, during Autumn 1969 and Spring 1970. Due to technical difficulties, sufficient material for study became available only from 17 cases of Burkitt tumour, and three other lymphomas. The biopsies were immediately placed into tissue culture medium at 37°C and cut into small blocks of tissue of about 1 mm^3. These were immersed in TC-199 containing 10% calf serum pH 7·35, and with 1 μCi 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 medium in each tube. These pieces (4 to 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 5 μ. After coating with film emulsion, the number of labelled cells was counted in each specimen after 2 weeks' exposure time. The specimens became rather oedematous during tissue culture, and labelling was found only in the superficial part of the piece [2]. The counting of labelled cells thus had to be limited to the periphery of the sections.

RESULTS

The 3H-thymidine labelling indices were used for calculating the line of best fit (Table 1). This table shows first primary Burkitt tumours with some clinical information, arranged in order of decreasing proliferation rates. Then follow four cases of relapsing Burkitt tumours, and finally the three cases of non-Burkitt lymphosarcomas. The slope of the regression line was used as an index of the rate of cellular proliferation. Figure 1 shows a graph of the results. The mean rate of cellular proliferation is 2·71% of all cells per hr, both in primary and in relapsing Burkitt tumours. This calculation is based on the assumption that the labelling index is a measure of cellular proliferation, i.e. that few cells die in G_2.

From this "birth-rate" of cells, the *potential doubling time* can be calculated. A rate of cellular proliferation of 2·71% of all cells per hr gives a potential doubling time of 25·5 hr in an exponentially growing cell population with no cell loss [3].

To measure the *actual doubling time*, we studied two other patients with cutaneous nodules of Burkitt tumour. Using the method described by Steel *et al.* [4] we calculated the actual doubling time from 4 tumours to be about 66 hr. The discrepancy between the potential doubling time and the actual doubling time is a measure of the rate of cell loss. It can be calculated that in our Burkitt tumours the rate of cell loss was about 70% of the rate of cell renewal.

This calculation is based on the assumption that the growth fraction (cycling cells/total number of cells) was approximately 100% in this tumour. Since the cell cycle time was not known, it was not possible to obtain a precise value for the size of the growth fraction. By injecting tritiated thymidine continuously, and observing the labelling index, a rough estimate could be obtained. Two patients in stage IV (those in which we measured the tumour volumes) were given tritiated thymidine every 6 hr, amounting to about 0·5 μCi per ml body volume per injection. Biopsies were taken at different time intervals and the number of labelled cells counted. The results are shown in Fig. 2. The form of this curve fits very well with the assumption that the growth fraction in this tumour is 90–100% of all cells [4].

Cultivation of biopsies *in vitro* from three patients with malignant lymphomas of non-Burkitt type gave the results shown in the bottom part of Table 1. The rate of cellular proliferation was estimated to be 3·78% of all cells per hr; which gives a potential doubling time of 18·3 hr. There is thus not much difference between the Burkitt tumour and other

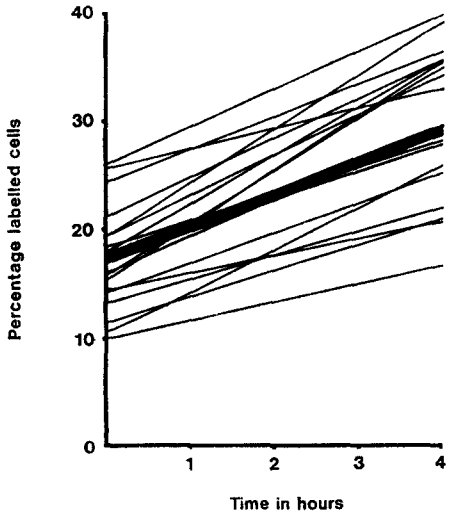

Fig. 1. Regression line through the labelling indices for 17 patients with Burkitt tumour (thin lines). The thick line is the average regression line. The slope of this line is a measurement of the rate of cellular proliferation of these tumours. For numerical values see Table 1.

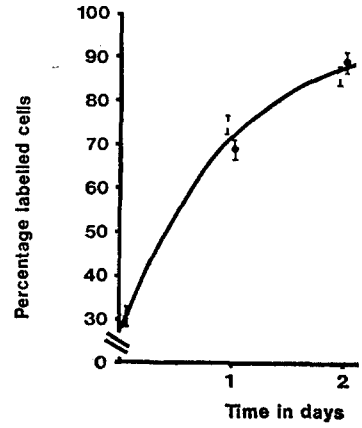

Fig. 2. Labelling indices from two patients in stage IV injected every 6 hr with tritiated thymidine. Biopsies were taken 1 hr after the first injection, and then on the first and the second day. The bars represent ± 2 standard errors of the mean.

Table 1. Clinical features, thymidine labelling indices, and potential doubling times in study patients

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	$Y = 15·49 + 5·38$	$X0·90$	12·9
5	8 F	II	Jaw	89+	89+	BL	23·05 ± 1·36	20·45 ± 1·92	32·45 ± 7·50	N.D.	$Y = 15·92 + 4·70$	$X0·74$	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	$Y = 19·30 + 3·90$	$X0·93$	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	$Y = 21·41 + 3·55$	$X0·85$	19·5
3†	4 F	III	Ovary	6	16	BL	20·80 ± 1·99	21·12 ± 1·71	25·81 ± 5·27	30·24 ± 2·97	$Y = 16·24 + 3·30$	$X0·95$	21·0
20	4 M	III	Jaw	28	60	BL	29·83 ± 3·15	N.D.	36·42 ± 5·52	N.D.	$Y = 26·53 + 3·30$	$X1·00$	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	$Y = 14·34 + 2·81$	$X0·97$	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	$Y = 24·56 + 2·72$	$X0·83$	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	$Y = 18·48 + 2·55$	$X0·61$	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	$Y = 11·61 + 2·36$	$X0·81$	29·4
19	11 F	III	Ovary	72+	72+	BL	20·85 ± 1·88	N.D.	22·45 ± 1·06	27·89 ± 1·49	$Y = 18·05 + 2·13$	$X0·88$	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	$Y = 22·55 + 1·42$	$X0·92$	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	$Y = 14·37 + 0·99$	$X0·60$	70·0
				Mean			21·84 ± 1·89	24·03 ± 1·50	27·11 ± 2·78	29·85 ± 2·83	$Y = 18·93 + 2·71$	—	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	$Y = 21·24 + 5·38$	$X0·95$	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	$Y = 11·10 + 3·64$	$X0·92$	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	$Y = 13·76 + 2·19$	$X0·77$	31·7
10	12 F	III	Ovary	20	129+	BL(R)	14·53 ± 1·00	10·86 ± 1·01	11·65 ± 1·23	20·19 ± 1·24	$Y = 9·87 + 1·77$	$X0·54$	39·2
				Mean			17·03 ± 1·33	19·09 ± 2·31	21·82 ± 1·50	25·16 ± 1·59	$Y = 13·99 + 2·71$	—	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	$Y = 22·61 + 5·28$	$X0·93$	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	$Y = 19·26 + 4·99$	$X0·86$	13·9
15	3 M	I	Jaw	40	52+	LSA	15·94 ± 5·93	N.D.	19·54 ± 1·27	24·08 ± 3·51	$Y = 12·96 + 2·58$	$X0·97$	26·9
				Mean			21·99 ± 4·30	33·18 ± 2·63	30·24 ± 4·21	35·58 ± 3·41	$Y = 20·79 + 3·78$	—	18·3

* Weeks from initial therapy.

† Patient 14 is the relapse in the same patient as No. 3.

Abbreviations: N.D. = not done; BL = Burkitt lymphoma; LSA = lymphosarcoma (poorly differentiated); R = relapse; r = correlation coefficient.

Some clinical data and the labelling indices of fresh biopsies from patients with Burkitt tumour, and patients with non-Burkitt undifferentiated lymphosarcomas, after 1, 2, 3 and 4 hr *in vitro* respectively. The labelling indices are given for each patient ± standard error of the mean. On the bottom lines are given the mean values. The regression line through all the 4 observation points can then be seen together with the correlation coefficient. The last column gives the calculated potential doubling times.

lymphomas of non-Burkitt type with regard to these parameters. A reservation must of course be made due to the small number of cases analyzed, and the wide variation in the results.

DISCUSSION

Our measurements are in agreement with the kinetic studies previously performed on cell lines from Burkitt tumours. We have used pieces of organized tumour tissue. All studies hitherto [5–8] have revealed potential doubling times between 19 and 48 hr, a large growth fraction, and a high degree of cell loss.

The practical inference from these observations is that, if one observes a patient with such a tumour one particular day, and returns to the same patient the next day, one does not see the "same" tumour. Almost all the cells have been exchanged.

This rapid rate of cell turn-over in the tumour may explain kinetically the rapid response to cytostatic treatment. But since the rate of cellular proliferation seems to be similar, or even faster, in other undifferentiated lymphomas, our results cannot explain why Burkitt tumours seem to be more curable than other lymphomas. Immunological mechanisms may be responsible for this phenomenon.

Clearly, more information is needed to obtain a thorough knowledge of the cell kinetics of this tumour. Further studies using stathmokinetic methods and comparing the results from each patient with the relevant clinical data, are in progress and will be reported later.

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Stimulated Granulocytopoiesis in Mice Bearing Fibrosarcoma*

L. MILAS and I. BAŠIĆ†
Department of Biology, Institute "Rudjer Bošković",‡ and Central Institute for Tumours and Allied Diseases, Zagreb, Croatia, Yugoslavia

Abstract—*Bone marrow, spleen and blood from (CBAT6T6 × C57BL)F1 mice bearing transplantable fibrosarcoma contained more CFUs than the same tissues from normal mice. In all the tissues assayed the pool of CFUs that form myeloid colonies in the spleen of recipient mice was particularly enlarged. Marked granulocytosis, corresponding to a stimulated granulocytopoiesis, indicated an increased release of differentiated cells into peripheral blood. Fibrosarcoma also decreased the immunological reactivity of host's spleen toward allogeneic tissue. These effects are explained in terms of a granulocytopoiesis-promoting factor secreted by the tumour, by "consumption" of granulocytes in the tumour, or by antigenicity of the tumour.*

INTRODUCTION

ALTERED haematopoiesis has frequently been found in animals as well as in patients bearing solid tumours that do not involve the haematopoietic tissue [1–4]. We have reported that mice with the fibrosarcoma had granulocytosis in the peripheral blood and that their bone marrow contained more colony-forming units (CFUs) than the marrow from normal mice [5, 6]. The present study deals with changes in haematopoietic activity that exist not only in the bone marrow of mice with the tumour, but in the spleen, lymph nodes and blood as well.

MATERIAL AND METHODS

Female (CBAT6T6 × C57BL)F1 hybrid mice 9–12 weeks of age were irradiated with 800 rads of X-rays [6] and injected intravenously with the bone marrow, spleen, lymph node or peripheral blood cells from syngeneic animals, normal or bearing a transplanted fibrosarcoma of about 20 mm in diameter. The tumour was induced by subcutaneous inoculation of 6×10^6 viable cells 20 days earlier. Cell suspensions from the tumour and haematopoietic organs were prepared as previously described [6, 7]. Haematopoietic colonies produced in the spleens of recipients were counted 8 days after the cell injections [6].

Graft-versus-host activity was assessed by weighing the spleen of A/H mice 6 days after irradiation with 500 rads of X-rays and injection of 50×10^6 cells to be assayed.

Experimental groups consisted of 6–12 mice. The results were evaluated according to the Student's *t*-test.

RESULTS

As compared with normal mice, the mice with the fibrosarcoma had 2·5 times more leukocytes in the peripheral blood (this was essentially due to granulocytosis) and 3 times more cells in their enlarged spleens, but their femurs contained 30% less bone marrow cells (Tables 1 and 2). Each of these tissues produced relatively more spleen colonies than the same tissues of normal mice, but the absolute number

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†Present address: Department of Animal Physiology, Faculty of Natural Sciences and Mathematics, University of Zagreb, Zagreb, Croatia, Yugoslavia.
‡Postal address: Institute "Rudjer Bošković", 41001 Zagreb, P.O. Box 1016, Croatia, Yugoslavia.

Table 1. *Mean number (M) of total leukocytes, granulocytes and lymphocytes in* 1 mm^3 *of blood from normal mice and mice bearing fibrosarcoma of* 20 *mm in diameter.* SE = *standard error.*

	Leukocytes M ± SE (× 10^3)	Granulocytes M ± SE (× 10^3)	Lymphocytes M ± SE (× 10^3)
Mice with tumour	24·5 ± 2·1	11·4 ± 1·1	12·9 ± 1·7
Normal mice	9·0 ± 1·0	0·7 ± 0·2	8·2 ± 1·0
P (*t*-test)	< 0·001	< 0·001	< 0·05

Table 2. *Mean number of nucleated cells in the bone marrow and spleen from normal mice and mice bearing fibrosarcoma of* 20 *mm in diameter*

	Cellularity of the femur M ± SE (× 10^6)	Spleen weight M ± SE (mg)	Cellularity of the spleen M ± SE (× 10^6)
Mice with tumour	10·1 ± 0·3	300 ± 30	330 ± 20
Normal mice	14·5 ± 1·1	70 ± 1·2	127 ± 8
P (*t*-test)	< 0·01	< 0·001	< 0·001

Table 3. *Mean number of colony-forming units (CFUs) in the bone marrow, spleen, lymph nodes and blood from normal or fibrosarcoma-bearing mice*

	Host Mean number of haematopoietic colonies produced in the spleen by				Donor Calculated* absolute number of CFUs in		
	5×10^4 bone marrow cells M ± SE	5×10^5 spleen cells M ± SE	5×10^6 lymph node cells	5×10^6 blood cells M ± SE	Femur M ± SE	Spleen M ± SE	1 ml of blood M ± SE
Mice with tumour	19·8 ± 0·9	18·3 ± 0·8	< 1	25·1 ± 1·4	23,600 ± 800	71,600 ± 4,900	720 ± 80
Normal mice	12·5 ± 0·8	2·5 ± 0·5	< 1	3·8 ± 0·9	21,400 ± 1,600	3,700 ± 200	40 ± 4
Ratio $\frac{\text{mice with tumour}}{\text{normal mice}}$	1·6	7·3		6·6	1·1	19	18
P (*t*-test)	< 0·001	< 0·001		< 0·001	N.S.†	< 0·001	< 0·001

*On the basis of cellularity and CFU settling efficiency in the spleen (*f* value) of 17% [8].
†Not significant.

Table 4. Myeloid "commitment" of CFUs in the bone marrow, spleen and blood from normal or fibrosarcoma-bearing mice

	Host			Donor		
	Mean number and percentage of myeloid colonies produced in the spleen by			Calculated* absolute number of CFUs that form myeloid colonies in host's spleen		
	5×10^4 bone marrow cells	5×10^5 spleen cells	5×10^6 blood cells	Femur M ± SE	Spleen M ± SE	Blood (1ml) M ± SE
Mice with tumour	5·0/19·8† (28%)	3·9/18·3† (21%)	10·0/25·1† (40%)	6,600 ± 220	15,000 ± 1000	290 ± 30
Normal mice	2·4/12·5 (19%)	0·1/2·5 (4%)	1·2/3·8 (31%)	4,060 ± 310	150 ± 9	13 ± 1·3
Ratio $\frac{\text{mice with tumour}}{\text{normal mouse}}$				1·6	100	22
P (*t*-test)				< 0·001	< 0·001	< 0·001

*On the basis of cellularity and CFU settling efficiency in the spleen (*f* value) of 17% [8].
†Mean numbers of myeloid colonies over total colonies per spleen.

of CFUs was elevated only in the spleens and blood, not in the marrow (Table 3). The lymph nodes contained negligible amounts of CFUs.

Histological analysis of the spleen colonies showed that the bone marrow, spleen, and blood cells from tumour-bearers produced a high percentage of myeloid colonies (Table 4). If we estimate the absolute number of CFUs giving myeloid colonies, it becomes clear that, for instance, the spleens from mice with tumours contained 100 times more "myeloid" CFUs than the spleens from normal mice.

Promoted haematopoiesis resulted in diminished immunological reactivity of the spleen cells of tumour-bearing mice. Injected into sublethally irradiated allogeneic mice, these cells produced no graft-versus-host reaction (Table 5).

Table 5. Mean spleen weight in 500 rads irradiated A mice injected with 50×10^6 spleen cells from normal or fibrosarcoma-bearing (CBAT6T6 × C57BL)F1 mice

Donors of spleen cells	Spleen weight M ± SE (mg)	P (*t*-test)
Mice with tumour	34 ± 2·0	N.S.*
Normal mice	49 ± 5·6	< 0·01
None	28 ± 2·3	

*Not significant.

DISCUSSION

Mice bearing the fibrosarcoma had more CFUs in their spleens, blood and bone marrow than normal mice. Most of these CFUs formed myelopoietic colonies, although the splenic environment actually favours differentiation towards erythrocytopoiesis [9].

In the bone marrow of mice with the tumour, the proliferation of CFUs was evidently limited by the available space, but in the spleen they could easily expand as their number grew. The increased number of CFUs in the blood can be attributed to their migration from the bone marrow to the spleen, as well as to an increased release from both. Such a migration was observed by Rencricca *et al.* under conditions of stimulated haematopoiesis [10]. Granulocytes were also released at an increased rate. This produced granulocytosis in the peripheral blood and, consequently, hypocellularity of the bone marrow.

Promoted haematopoiesis in the spleen decreased the ability of spleen cells to produce splenomegally in allogeneic, sublethally irradiated mice, probably because the injected inoculum of spleen cells consisted predominantly of immunologically non-reactive haematopoietic cells.

These experiments leave unanswered the question what stimulated the proliferation of CFUs and the release of CFUs and granulocytes into peripheral blood. Delmonte showed

that an extract from transplantable mammary carcinoma of mice contained a granylocytopoiesis-promoting factor able to augment the release of granulocytes and to stimulate proliferation of CFUs in the bone marrow [11, 12]. A factor that accelerates mitosis and increases the number of haematopoietic colonies *in vitro* has been found by Metcalf *et al.* in the serum and urine of leukaemic (and normal) individuals [13–15]. Our preliminary results, that the serum from mice with the fibrosarcoma induces granulocytosis in normal syngeneic mice, suggest an existence of a similar factor that would specifically stimulate granulocytopoiesis.

Other factors, however, have to be taken into consideration: necrosis present in the growing tumour, might have non-specifically stimulated proliferation of granulocytes; or the large tumour mass "consumed" increased amounts of granulocytes and thus stimulated their production by a feedback mechanism [16]. Further, this tumour contains tumour-specific antigen(s) [7] which, like other foreign materials, could expand the content of colony-forming units through an antigenic effect [17, 18].

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The Specificity of Carcino-Foetal Antigens of the Human Digestive-Tract Tumours*

F. MARTIN, M. S. MARTIN, M. BORDES and C. BOURGEAUX
I.N.S.E.R.M., U. 45 and Laboratoire de Médecine Expérimentale, Faculté de Médecine, 21, Dijon, France

Abstract—*A carcino-foetal antigen, identical to Gold's carcino-embryonic antigen, may be demonstrated by the immunization of rabbits with human colonic adenocarcinoma. After absorption by homologous normal tissue, the antiserum reacts with saline extracts of digestive tumours or foetal gut, but not with non-cancerous adult tissue. However, more sensitive techniques (precipitation and precipitin-inhibition reaction using perchloric extracts, immunofluorescence, radioimmunoassay) can detect small amounts of the same antigen in non-cancerous adult colon. Therefore, the carcino-embryonic antigen seems not to be cancer specific, although definite quantitative difference may be demonstrated between cancerous and non-cancerous intestine. From a practical point of view, this difference is sufficient to be useful for diagnostic purposes.*

In the same way, two other carcino-foetal antigens of the human digestive system, alpha-foetoprotein of hepatoma and foetal sulphoglycoprotein of gastric carcinoma, seem to have been detected where foetal or cancerous conditions do not prevail.

In the past few years, a particular group of proteins has been found to be related to cancers of the digestive system. These proteins, normal components of foetal digestive organs, seem to be absent in adult tissues, but are detectable in carcinoma involving these organs. Hence, they can be referred to as carcino-foetal protein or, as immunological techniques are necessary for their detection, as carcino-foetal antigens. Among carcino-foetal antigens of the digestive system, three have been more extensively investigated: alpha-foetoprotein related to hepatoma, foetal sulphoglycoprotein to neoplasm of the stomach, and carcino-embryonic antigen to colonic tumour.

(1) *Alpha-foetoprotein* is one of the main components of foetal serum. In normal circumstances, it decreases in amount at the end of the foetal life and it is no longer detectable a few weeks after birth. Abelev *et al.* [1] were the first to demonstrate its reappearance associated with experimentally induced hepatoma in mice. In 1965, Tatarinov [2] detected alpha-foetoprotein in serum of human patients with hepatoma.

(2) In 1965, Gold and Freedman [3] found a particular antigen in carcinoma of colon or rectum. This antigen was also detected, at lower concentrations, in cancers of stomach, pancreas or liver, and in the foetal digestive system. Hence, it was named *carcino-embryonic antigen* (CEA) of the human digestive system [4].

(3) *Foetal sulphaglycoprotein* was found by Häkkinen [5] in 1966, in the acid glycoprotein fraction of the gastric juice of patients with gastric cancer. It was detected by immunofluorescence in gastric tumours and in gastric mucous cells of the foetal stomach [6].

Carcino-foetal antigens are promising for cancer investigation: they can be, and are at at least for alpha-foeto protein, a useful tool for

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diagnosis of digestive tumours; they can have a major theoretical significance for aetiology of carcinogenesis; they could even be potential targets for spontaneously or artificially induced immunological defence mechanisms. Fulfilment of these promises depends partly on the answers to the following questions:

—are carcino-foetal antigens really cancer-specific,

—are they autoantigens, i.e. immunogenic for the tumour-bearing host?

This work will deal with these two questions, studying more particularly carcino-embryonic antigen, of which we have a personal experience.

ARE CARCINO-FOETAL ANTIGENS REALLY CANCER-SPECIFIC?

It is always difficult to establish the absolute cancer-specificity of a human component; it would imply that this component is completely lacking in non-cancerous tissues, even in extremely small amounts. Of course, the reliability of this negative result depends on the sensitivity of the methods used for detecting the "specific" protein.

The cancer specificity of foetal proteins is particularly questionable, as genetic material necessary for their synthesis is encoded in normal cells. The repression of this synthesis could thus be only partial: for instance, the switch from foetal haemoglobin *F* to adult haemoglobin *A* is not complete, the former persisting in traces throughout adult life.

(a) *Alpha-foetoprotein*

From a practical point of view, alpha-foetoprotein appears to be cancer specific [7]: the one condition, besides hepatoma, where alpha-foetoprotein is frequently found in an adult, is in embryonic tumours of the testes. However, that alpha-foetoprotein might reappear outside malignancy is supported by experimental evidence: in adult mice, alpha-foetoprotein synthesis may be induced, without hepatoma, by partial hepatectomy or exposure to carbon tetrachloride [1].

In humans, alpha-foetoprotein has occasionally been found in a few cases of adult patients with liver cirrhosis or viral hepatitis [8–10]: even if some of these cases could be explained by association with a latent hepatoma, the question of complete cancer-specificity of alpha-foetoprotein remains open. Furthermore, using a highly sensitive immuno-autoradiography technique, Abelev has reported a transitory detection of alpha-foetoprotein in 9 out of 54 patients with viral hepatitis [11].

(b) *Foetal sulphoglycoprotein*

By immunofluorescence, Häkkinen has found foetal sulphoglycoprotein in most of the specimens of gastric tumours [6]. However, the antigen was not localized to cancerous cells but was also found in morphologically normal cells, at some distance from the cancer. Furthermore, foetal sulphoglycoprotein was present in 4 out of 29 stomachs removed for peptic ulcer.

(c) *Carcino-embryonic antigen*

According to Gold, "the carcinoembryonic antigen of the human digestive system is a tumour-specific constituent of all adenocarcinomas arising from the entodermally derived epithelium of the digestive system. It is absent from all other adult human tissues, normal, cancerous, or otherwise diseased. CEA is thus designated because it is also in the digestive organs of the human embryo and foetus in the first 2 trimesters of gestation" [12].

Gold's claims about the qualitative cancer-specificity of CEA were supported by reactions obtained with heterospecific antiserum; this serum was obtained by immunization of rabbits with saline extracts of colonic tumours. Antibodies directed against non-cancer distinctive components were removed by absorption with extracts of the normal colons distal to the tumours used for immunization. According to Gold, this serum gives a single precipitin band with saline extracts of colonic tumours, but not with saline extracts of normal adult tissues; it can be absorbed by saline extracts of digestive adenocarcinomas, but not by saline extract of normal colon [3, 4].

Gold's conclusions about cancer-specificity of CEA have also been confirmed in similar works by Kronman *et al.* [13] and Kleinman *et al.* [14]. However, several experiments performed in our laboratory do not support the hypothesis of a complete cancer-specificity of CEA. In fact, small but significant amounts of CEA were demonstrated in non-cancerous colonic mucosa. Before proceeding to this point, however, it will be necessary to make some comments concerning methodology.

(1) Specific rabbit anti-CEA serum was prepared according to Gold [3]. Specificity of serum was checked by immunodiffusion: it gave a common precipitin band with saline extracts of different colonic tumours, but did not react with saline extracts of non-cancerous colon [15]. When compared with 2 samples of anti-CEA serum, kindly supplied by Dr. Gold and Drs. von Kleist and Burtin, a reaction of identity was obtained between the three serums.

(2) As demonstrated by Gold [16], CEA may

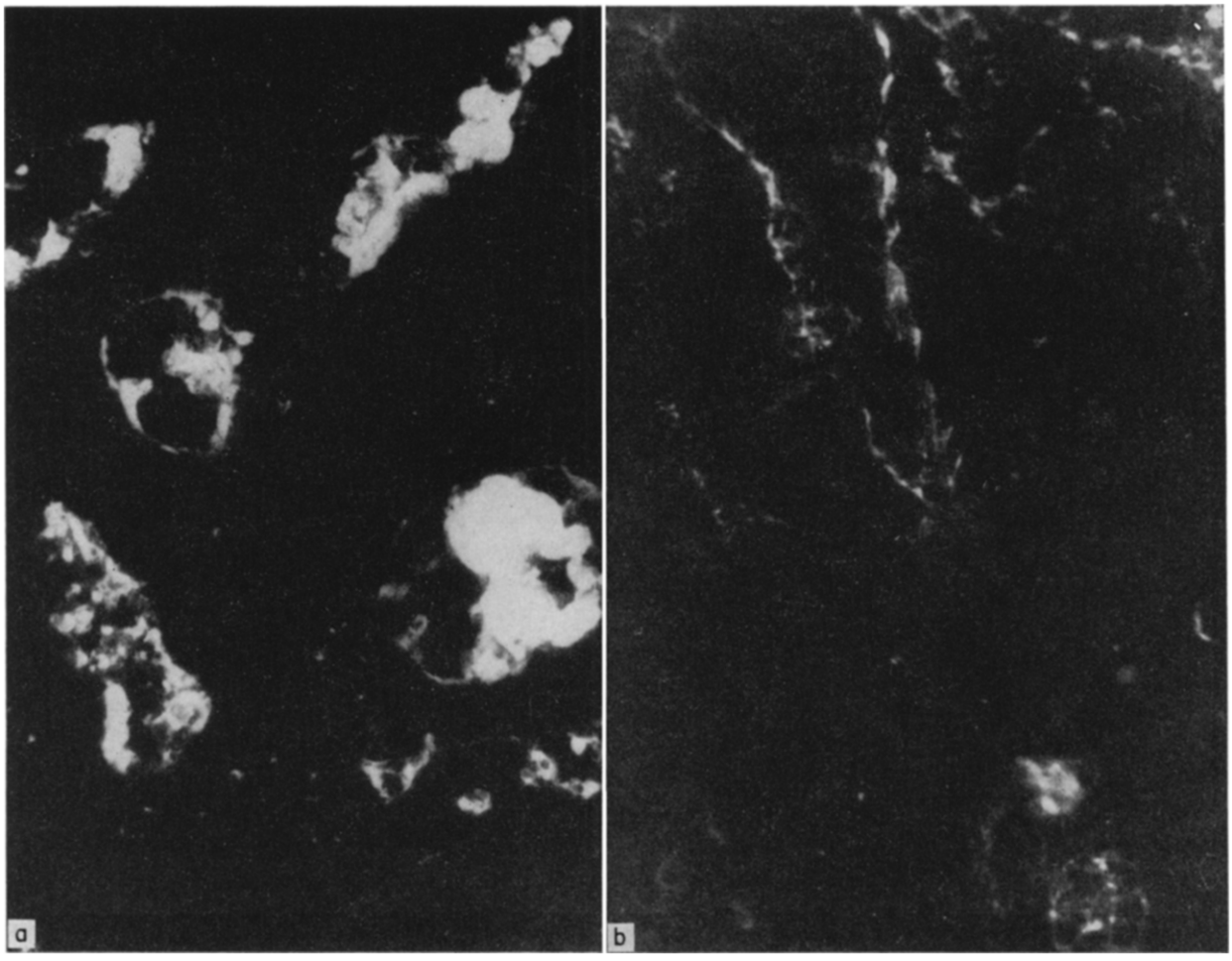

Fig. 1. Localization of CEA by immunofluorescence. A, Colonic adenocarcinoma. B, Morphologically normal mucosa, taken at distance from the tumour.

(to face p. 316)

be concentrated from saline extracts using its solubility in perchloric acid. Perchloric extracts were thus prepared from different normal or cancerous tissues.

(3) CEA was further purified from perchloric extract of liver metastasis of colonic tumour, according to a modification of the method described by Krupey *et al.* [16] (which included 2 separations on sephadex G-200, preparative electrophoresis on Pevikon, and preparative chromatography on Sepharose 4B). On electrophoresis, purified CEA gives one precipitation line with unabsorbed anti-colonic tumour serum.

(4) Radioimmunoassay of CEA was first performed according to Thomson *et al.* [17]. However, addition of perchloric extract of human normal serum in the standards was proved to interfere with the results. Suppression of this extract led to excessive non-specific precipitation of labelled CEA. Satisfying results were obtained by adding a small amount (0·5 mg) of perchloric extract of normal human saliva in standards and in tested samples.

lines formed with these extracts showed reactions of complete identity between themselves and saline extract of colonic tumour.

Precipitin-inhibition reaction. Precipitin inhibition reaction was performed according to Gold [4], but perchloric extracts were used instead of saline extracts. Inhibition of the precipitin line was obtained with most of the samples of non-cancerous colonic mucosa if perchloric extract was added to antiserum in sufficient amounts (80–160 mg/ml of antiserum). The results are summarized in Table 1. Precipitin-inhibition was achieved with far smaller amounts of perchloric extracts of colonic tumours (1·25–20 mg/ml of antiserum). On the other hand, it was not possible to inhibit the precipitation with perchloric extracts of non-cancerous gastric mucosa (6 samples) or normal liver, pancreas, kidney or lung [18].

(2) *Immunofluorescence*

Immunofluorescence studies were performed by the method described by von Kleist and Burtin [18], using the Coons indirect (or

Table 1. *Detection of CEA by precipitin-inhibition reaction in perchloric extracts of colonic adenocarcinoma and non-cancers colonic mucosa.*

Minimum amount of extract inhibiting 1ml antiserum (mg)	Cases	
	Adenocarcinoma of colon or rectum	Non-cancerous colonic mucosa
1·25	1	0
2·5	1	0
5	5	0
10	4	0
20	4	0
40	0	0
80	0	4
160	0	7
more than 160 mg (negative result)	0	1

The concentration of CEA is estimated by the smaller amount or perchloric extract able to inhibit the specific antibodies of 1 ml of rabbit antiserum.

We have used three methods for detecting CEA in various normal or cancerous organs: immunodiffusion, immunofluorescence and radioimmunoassay.

(1) *Immunodiffusion*

Direct Ouchterlony's technique. Whereas reaction cannot be demonstrated between specific anti-CEA serum and *saline* extracts of non-cancerous colonic mucosa, a precipitin line may be obtained if concentrated (4–8%) *perchloric* extracts of normal colon were used [15]. The precipitin

sandwich) method. In samples of colonic adenocarcinomas, a very bright fluorescence was localized at the apical pole of cancerous cells. The mucus contained in neoplastic glands was also intensely stained (Fig. 1A). At the present time, we have not yet had the opportunity to examine colon removed for disease other than intestinal tumour. However, a mild but significant fluorescence was found in morphologically normal cells, taken more than 10 cm from the edge of the tumour. (Fig. 1B). As on cancerous cells, fluorescence was localized

to the outline of the cells, chiefly on the apical pole. A similar pattern has been previously reported by von Kleist and Burtin [18].

(3) *Radioimmunoassay*

Radioimmunoassay of CEA has been performed on pooled perchloric extracts of different organs. Results are given in Table 2. CEA may

Table 2. Radioimmunoassay of CEA in perchloric extracts of different organs

Normal liver ..... less than	0·1 mg/g
Normal stomach ..... less than	0·1 mg/g
Gastric cancer	1·5 mg/g
Normal colon	2·0 mg/g
Foetal gut	2·5 mg/g
Colonic cancer	75 mg/g
Colonic cancer (liver metastasis)	100–200 mg/g

The results are given in mg of CEA/g of perchloric extract.

be detected in mucosa of colon removed for non-cancerous diseases, although in amount about 50 times weaker than in colonic cancer. On the other hand, CEA cannot be found in perchloric extracts of normal stomach or liver. Curves obtained with increasing concentrations of purified CEA, perchloric extracts of colonic tumours or perchloric extracts of non-cancerous colon were parallel, indicating the presence of antigen showing identity of immunological behaviour (Fig. 2).

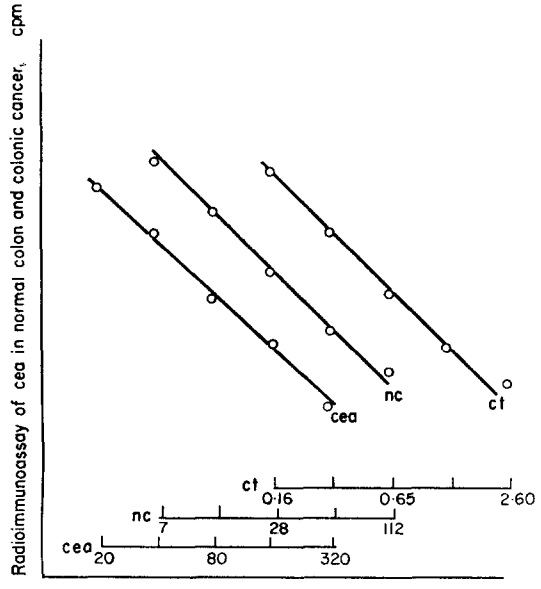

Fig. 2. Radioimmunoassay of CEA in perchloric extracts of colonic cancer or non-cancerous colon. Curves obtained with increasing concentrations of purified carcino-embryonic antigen (cea), *perchloric extracts of colonic tumours* (ct) *and perchloric extracts of non-cancerous colon* (nc) *are parallel, indicating the presence of antigen showing identity of immunological behaviour. Concentration are expressed in nanograms for purified CEA, in micrograms for perchloric extracts.*

From these experiments, it may be concluded that CEA, as foetal sulphoglycoprotein and probably alphafoetoprotein, is not qualitatively cancer-specific. However, there is a major quantitative difference in concentration of carcinofoetal antigens between normal and cancerous tissues. So these foetal proteins can be referred to as "cancer-related" or "cancer-distinctive" [19], if not "cancer-specific".

ARE CARCINO-FOETAL ANTIGENS IMMUNOGENIC FOR THE TUMOUR-BEARING HOST?

The carcino-foetal proteins are often referred to as antigens, but only because they are identified by an immunological technique: heteroimmunization of rabbit or goat. But this capacity to elicit antibodies in animals does not mean that these proteins are immunogenic for the tumour-bearing patient.

As far as we are aware, there is no report of any immunological mechanism directed against alpha-foetoprotein or foetal sulphoglycoprotein. The opposite would be surprising as these two proteins are still detectable near the time of birth and can then induce immunological tolerance.

On the other hand, Gold has reported that circulating antibodies against CEA could be found in serum of about 70% of the patients with non-metastatic cancer of the digestive system [20]. The antibodies were also present in pregnant women. They were absent from most patients with non-cancerous disease, with cancers other than those of the digestive tract, or with metastatic cancers of digestive organs. Binding of antibodies on a large cancerous mass could explain this latter result.

However, resuming Gold's technique, von Kleist and Burtin could not find anti-CEA antibodies in serum of patients with digestive tumours [21]. Furthermore, specificity of antibodies demonstrated by Gold is questionable: in the BDB passive haemaglutination technique, red cells were sensitized by perchloric extract of colonic cancer. Besides CEA, this extract contains several other components, with which autoantibodies may react.

Using ^{125}I-labelled purified antigen, we have tried to detect circulating antibodies specifically directed against CEA. Radioactivity bound to these antibodies is measured after precipitation by half-saturated ammonium. Non-specific precipitation is lowered by the addition of salivary glycoprotein. This technique is sensitive, as anti-CEA antibodies could still be detected in a specific rabbit anti-serum at a dilution of

1/204,800. There was no significant difference between the group of patients with digestive tumours and patients without cancer (Fig. 3). Only three patients from the former group and one from the latter had a serum with an antigen-binding capacity greater than that of specific rabbit antiserum at a dilution of 1/102,400. A patient, whose serum was taken a few days after colonic cancer removal, had an antigen binding activity approximately equal to that of rabbit antiserum at a dilution of 1/22,000.

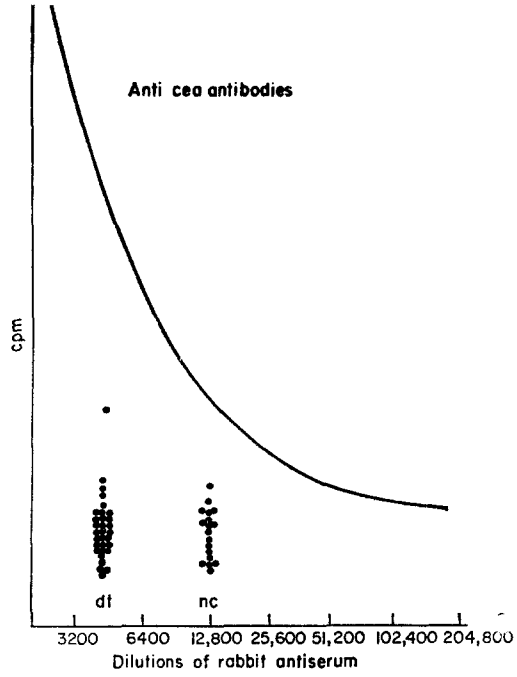

Fig. 3. Radioassay of autoantibodies directed against CEA. The curve represents the radio-labelled CEA bound to antibodies for increasing dilutions of rabbit specific antiserum. Each point represents the result obtained with one individual serum, at the dilution of 1/5. dt: *patients with digestive tumours.* nc: *patients without cancer.*

PRACTICAL USE OF CARCINO-FOETAL ANTIGENS IN DIAGNOSIS OF HUMAN DIGESTIVE TUMOURS

That carcino-foetal antigens are neither cancer-specific, nor probably immunogenic, is indeed deeply disappointing. But it does not prevent them from being of practical value in the diagnosis of digestive cancers.

Alpha-foetoprotein

The immunodiffusion technique of Ouchterlony allows for detection of alpha-foetoprotein in 40–80% of the patients with hepatoma. Apart from the exceptional cases referred to above, it does not detect the antigen in patients without neoplastic disease. The chief limitation of the method is the high percentage of alpha-negative patients with hepatoma. It may be expected that more sensitive tests (immuno-auto radiography, haemaglutination, radio-immunoassay) would yield a higher percentage of positive reaction; but these methods would also detect alpha-foetoprotein in some patients without hepatoma [11].

Foetal sulpho-glycoprotein

This has been found by Häkkinen in the gastric juice of about 96% of patients with a gastric cancer. It was never found in healthy young controls, but could be detected in 12% of patients with non-cancerous gastric disease, chiefly atrophic gastritis and peptic ulcer [22]. These "false positive" cases require careful interpretation of the results. However, the high percentage of positive reactions in patients with gastric cancer give to this test a considerable practical value, if it is proved not be related especially to this cancer in Finland.

Carcino-embryonic antigen

In 1969, Thomson and Gold published very promising results regarding the detection of circulating CEA by radioimmunoassay [17]. Circulating CEA was found in most of the cases of preoperative, residual, recurrent or metastatic cancers of the large intestine. It was never detected in the other cases, except for a few patients with non-intestinal digestive tumours. One hopes that Gold's results will lead to a very useful test in the diagnosis and prognosis of the intestinal cancers.

However, somewhat different results have recently been published by Moore and Zamcheck [23]. Using Gold's technique and reagents, they found that CEA was never detectable in controls but was present in most patients with colonic tumours. However, the antigen was also detected in the serum of a high percentage of patients with other digestive cancers, non-digestive tumours, or non-cancerous diseases of liver or kidney.

We have only a limited experience with radioimmunoassay of circulating CEA. We have used the modification of Thomson's technique, as described above, adding salivary glycoproteins instead of perchloric extracts of normal serum in standards. With this technique, we have found CEA in all the serums tested. In control patients without cancer CEA level was always inferior to 18 nanograms per ml of serum. CEA level was above this limit in 70% of patients with digestive cancers (Fig. 4).

Detection of CEA in all the sera, even from normal control, is in disagreement with Gold's and Zamcheck's data. It could be explained by

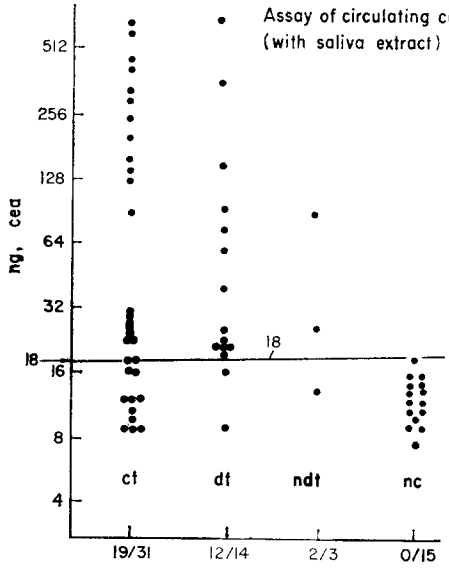

Fig. 4. Radioimmunoassay of circulating CEA. The results are expressed in nanograms of CEA per ml of serum. ct: *patients with colonic adenocarcinoma.* dt: *patients with other digestive cancers.* ndt: *patients with cancers localized outside the digestive system.* nc: *patients without cancer, and healthy controls.*

the voluntary omission of perchloric extract of normal human serum in the standard curve; it may be supposed that addition of this extract automatically lowers the result by subtracting CEA normally contained in serum.

In any case, data collected by the three groups of investigators demonstrate that radioimmunoassay of circulating CEA may have a clinical value in the diagnosis of the digestive tumours.

So, even if not completely cancer-specific, the three major carcino-foetal antigens may be a useful tool for the detection of cancers of the digestive tract. It is even possible that they may some day be a point of attack for immunological treatment. Indeed, as Southam pointed out [24]: "it might not be necessary for a 'cancer-specific' antigen to be quantitatively abnormal in order to be of importance in cancer. A major quantitative difference in concentration of an antigen between normal and neoplastic cells might be useful in diagnosis or as a point of attack in therapy."

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Oestrogen Receptors in Hamster Tumours

A. W. STEGGLES* and R. J. B. KING

Department of Hormone Biochemistry, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, England

Abstract—*Cytoplasmic 8 and 4S, and nuclear 5S oestradiol receptors have been detected in oestrogen dependent primary, oestrogen-dependent transplant and oestrogen-independent transplant tumours. There was no correlation between receptor content of the different tumours and their oestrogen responsiveness. The association of oestradiol with the nuclear receptor is acid-labile and oestrogen-specific. Evidence from protamine sulphate, N-ethyl maleimide and KCl studies suggest that the 8 and 4S receptors are related, and are similar to those found in rat uterine cytoplasm.*

Normal hamster uterus has maximal number of cytoplasmic binding sites/cell on day 2 of the cycle. Immature or ovariectomised uteri contained 8S cytoplasmic receptor whilst mature uteri contained both 8 and 4S receptors. The proportions of these receptors depended on the stage of the oestrus cycle. Nuclei contained 5S receptor.

INTRODUCTION

CONSIDERABLE evidence has now been accumulated on the presence of oestrogen receptors in normal tissues such as rat uterus [1–4], but only one detailed study has been carried out on tumour tissue. King *et al.* [5] demonstrated the presence of cytoplasmic 8 and 4S, and nuclear receptors in the oestrogen-dependent dimethylbenzanthracene (DMBA) induced rat mammary tumour by use of sucrose gradient analysis.

Oestrogen-dependent hamster kidney tumour retained oestradiol to a greater extent than the independent one [6], which suggested that the former behaved as an oestrogen target tissue. Consequently it was decided to investigate these tumours by sucrose gradient analysis and subcellular fractionation studies. This paper describes the subcellular localization of [6, 7-^{3}H] oestradiol, as well as the detection and some of the physical properties of both cytoplasmic and nuclear receptors in these tumours.

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*Now at the Department of Obstetrics and Gynecology, Vanderbilt University Medical School, Nashville, Tennessee 37203, U.S.A.

MATERIAL AND METHODS

Animals

Hamsters were obtained from the Imperial Cancer Research Fund breeding colony at Mill Hill. Those undergoing oestrogen treatment were implanted subcutaneously with pellets of either stilboestrol (20 mg) or oestradiol (15 mg) at 3 monthly intervals for the duration of the experiment [6]. Unless stated otherwise, all animals were used with pellets *in situ*. After 9 to 12 months of treatment animals were killed and, if kidney tumours were present, they were separated from the surrounding kidney tissue, and dissected free of necrotic areas. These are referred to as dependent primary tumours. Only 29 tumours greater than 10 mg wet weight were obtained from 650 oestrogen treated hamsters. Most of the hamsters died as a result of the oestrogen treatment.

Transplanted renal tumours

Four types were used. (1) Dependent transplanted tumour. The dependent primary tumour was transplanted into animals which had been given a stilboestrol pellet three months earlier [6]. (2) "Independent" tumour from a primary tumour by successive serial trans-

plantation. This has been described previously [6] and was originally obtained from Dr. H. J. G. Bloom and Mr. B. C. V. Mitchley of the Chester Beatty Research Institute. Unless stated otherwise it was transplanted into intact male hamsters and in these experiments it had been transplanted 148 to 160 times. It has been described as a fully independent tumour [7] but this is incorrect as it grows faster in female than in male hamsters [8; also our own unpublished observations] and growth is retarded by some antioestrogens [8]. It is however much less dependent on oestrogen than the primary or dependent transplanted tumours as these will not grow in male hamsters. (3) The 6393 independent tumour. This was an autonomous variant kindly sent to us by Dr. H. Kirkman. It grows equally well in male and female hamsters and was transplanted into male animals. (4) RCA_1 tumour. This was originally an oestrogen spontaneous unresponsive renal adenocarcinoma and was kindly given to us by Dr. H. Skipper. It was transplanted into male hamsters.

Labelling of tissues

The preparation and purification of [6, 7-^{3}H] oestradiol (specific activity 216 to 235 $\mu c/\mu g$ and [1, 2-^{3}H] dihydrotestosterone (specific activity 137 $\mu c/\mu g$) have been described previously [9, 10]. The *in vivo* administration of [6, 7-^{3}H] oestradiol, and the extraction of radioactivity from tissues has also been described [6]. The hamsters were killed 0·5 hr after the administration of [6, 7-^{3}H] oestradiol, the tumours were excised and either used immediately or stored at −20°C until use. Under these conditions, 60 to 80% of the tritium is present as oestradiol [6]. Radioactive samples were counted in Packard liquid scintillation spectrophotometers, models 3003 and 3375. The phosphor contained 100 g naphthalene and 3·5 g of 2,5 diphenyl oxazole in 385 ml xylene, 385 ml dioxane and 230 ml absolute ethanol. Counting efficiency was 15 to 25%.

Sucrose gradients

Gradient centrifugation was carried out as described by Martin and Ames [11]. Gradients were prepared by layering 1·1 ml each of 20, 15, 10 and 5% sucrose (w/v) containing 10 mm Tris-HCl, 1·5 mM EDTA (Tris/EDTA) pH 7·4 into centrifuge tubes and storing at 4°C for 18 hr prior to use. Occasionally the sucrose solutions contained 0·3 M KCl, these are referred to as Su/KCl gradients. Samples to be analysed, usually 0·2 to 0·4 ml, were layered carefully onto the gradients, and centrifuged at 39,000 rev/min (125,000 **g** av) for 16 to 18 hr at 5°C. After centrifugation the bottom of the tube was pierced, 3-drop fractions collected and the radioactivity measured. For *in vitro* labelling, tissues were minced with scissors, defatted, and transferred to vials containing 5 ml Krebs phosphate buffer II [12] with 1 nM [6, 7-^{3}H] oestradiol. Incubations were at 4°C when cytoplasmic binding was required and at 37°C for nuclear binding. The buffer was replaced at 0·5 and 1 hr. After 2 hr the tissues were washed twice with 5 ml Tris/EDTA and homogenized in 2 ml of the same buffer, to give a 30% (w/v) homogenate. Either a Silverson homogenizer (Silverson Machines Limited, London, S.E.1.) or a Teflon–glass homogenizer was used. The homogenate was centrifuged at 10^5 **g** for 1 hr, to give a cytoplasmic fraction, and a 10^5 **g** "nuclear pellet". Aliquots of the cytoplasm were routinely taken for counting and protein estimations, and for gradient centrifugation. The 10^5 **g** pellet was gently homogenized in 1 ml of 0·3 M KCl/Tris/EDTA and left at 4°C for 10 min. After centrifugation at 10^5 **g** for 1 hr, a "KCl-soluble nuclear" extract was obtained, and a residual pellet. Aliquots of the "nuclear" extract were taken for counting, and gradient centrifugation. The residual pellet was digested in 5 ml 1 N NaOH, and aliquots taken for counting and DNA estimations.

All experiments involving sucrose gradients, including the killing and dissection of the animals were carried out at 4°C.

Protamine precipitation

The protamine precipitation technique was derived from the observation that polycations, like protamine and polylysine, can precipitate oestradiol receptors [5]. Aliquots (0·4 ml) of the cytoplasmic or "KCl-soluble" nuclear extracts were mixed with 0·2 ml 0·75% (w/v) salmine sulphate (British Drug Houses Limited) solution at 4°C for 5 min. After centrifugation at 10^3 **g** for 5 min, the precipitate was digested with 0·5 ml 1 N H_2SO_4. All the supernatant and acid digests were counted. In some instances an aliquot of the supernatant was layered onto sucrose gradients and centrifuged.

The use of protamine to determine binding constants of tumour cytoplasm

In these experiments tissues were incubated as before, but without added [6, 7-^{3}H] oestradiol. Aliquots (0·2 ml) of the cytoplasm were mixed with 0·2 ml of different concentrations of [6, 7-^{3}H] oestradiol and left at 4°C for 1 hr. Salmine sulphate solution (0·2 ml) was then added and the precipitate collected. The

radioactivity in the precipitate and supernatant fractions are referred to as "bound" and "free" oestradiol respectively [5]. A Scatchard plot was made [13] and the binding constants calculated.

Preparation of nuclei

Tissues were homogenized in 0·25 M sucrose containing 3 mM $CaCl_2$ (0·25 M Su/Ca) using either a Silverson or a Teflon–glass homogenizer. After filtration through a stainless steel sieve (100 mesh), the homogenates were centrifuged at 700 **g** for 10 min. The "nuclear" pellet was resuspended in 4·5 ml of 2·2 M Su/Ca and centrifuged at 5×10^4 **g** for 30 min to give purified nuclei [14]. These were washed once with 10 mM Tris-HCl pH 7·4.

Extraction of nuclei

(*a*) *NaCl solutions.* Clean nuclei were resuspended in 100 mM Tris-HCl pH 7·4. Aliquots (0·5 ml) were mixed with equal volumes of 0·2, 1·0 and 4·0 M NaCl and incubated at 0° for 10 min. After centrifugation at 3000 **g** for 10 min, aliquots of supernatant and pellet were taken for counting, DNA and protein estimations.

(*b*) *Buffer solutions.* Nuclei were incubated at room temperature for 10 min in 1 ml 0·15 M acetate–borate–cacodylate (ABC) buffer. ABC buffers between pH 3 and 9. After centrifugation at 3000 **g** for 10 min, aliquots of supernatant and pellet were taken for counting, DNA and protein estimations. This buffer was used to minimize effects due to changes in ionic strength or ionic species [15].

(*c*) *Enzymic digestion of nuclei.* Nuclei were resuspended in 100 mM Tris-HCl pH 7·4 and aliquots incubated at room temperature with either (*a*) 0·1 ml of 0·2% ribonuclease (Sigma Chemical Company) and 0·05 ml of 0·05 M $MgSO_4$, (*b*) 0·1 ml of 0·1% deoxyribonuclease (Sigma Chemical Company) or (*c*) 0·1 ml of 0·2% trypsin (Sigma Chemical Company) for either 5 min [(*a*) and (*b*)] or 10 min (*c*). After centrifugation at 3000 **g** for 10 min, aliquots of supernatants and pellets were taken for counting, DNA and protein estimations.

Sonication studies

Nuclei were resuspended in 2 ml 0·25 M sucrose containing 100 mM Tris-HCl pH 7·4, and sonicated at 20 kcycles for 30 sec using an MSE ultrasonic power unit. The suspension was centrifuged at 100 **g** for 5 min, and the supernatant centrifuged successively at 10^3 **g** for 10 min, and 10^3 **g** for 1 hr. Aliquots of supernatants and pellets were taken for counting and DNA determinations. Most of the chromatin is sedimented by this procedure [16].

DNA was measured by the diphenylamine reaction [17] with salmon sperm DNA (Sigma Chemical Company) as standard, and protein by the method of Lowry *et al.* [18] with crystallized bovine serum albumin as standard (Armour Pharmaceuticals Limited).

RESULTS

Studies on cytoplasmic and nuclear oestradiol binding using sucrose gradients

Liver, normal kidney and kidney from 9 month oestrogen-treated hamsters were labelled *in vitro* with [6, 7-^{3}H] oestradiol (see Material and Methods). After homogenization and centrifugation aliquots of the 10^5 **g** supernatant were layered onto 5–20% sucrose gradients. Hamster serum, bovine serum albumin (bsa), and immature rat uterine cytoplasm were used as reference markers of 4–5S, 4·6S [9] and 8S respectively. The results are shown in Fig. 1A–D. The sedimentation profile from the normal kidney (AI) showed little oestradiol binding whereas the oestrogen-treated kidney showed considerable binding in the 4S region, just behind bsa (AII). Hamster serum (CII) and bsa (CI) also showed oestradiol binding in the 4–5S region. It is probable that the 4S binding in liver (B) and the oestrogen-treated kidney was non-specific, as very little was precipitated by protamine (Table 1).

Sucrose gradient profiles were obtained from *in vivo* and *in vitro* labelled dependent primary, dependent transplanted and independent tumours. Both cytoplasmic and 0·3 M KCl "nuclear" extracts were studied. Only the *in vitro* cytoplasmic profiles are shown although the *in vivo* results were similar. The sedimentation values for the cytoplasmic receptors were similar. The sedimentation values for the cytoplasmic receptors were assessed as 8 and 4S, and the nuclear receptor as 5S on the basis of a comparison with bsa and immature rat uterus (Fig. 1D). The 4S binding in both the dependent and independent tumours was judged to be specific on the basis of its protamine precipitability, (Table 1) and its disappearance when the stilboestrol implant was present (Fig. 2AII). When the stilboestrol implant was removed 47 h prior to tumour excision, profiles showing oestradiol binding were obtained (Fig. 2AI). The profile for the "nuclear" extract from the dependent primary tumour showed a single 5S receptor (Fig. 2B). Similar profiles were obtained from the dependent transplanted tumour (Fig. 2CI and DI).

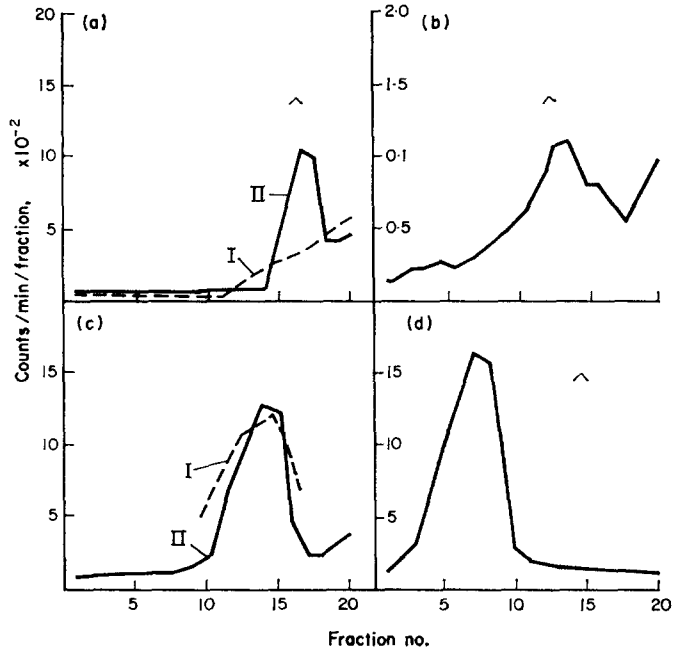

Fig. 1. *Sedimentation of cytoplasmic extracts on* 5–20% *sucrose gradients. Centrifugation for* 16 *hr at* 4°*C and* 39,000 *rev/min, with sedimentation from right to left. All tissues were labelled with* 1*nM* [6, 7–^3H] *oestradiol for* 1 *hr* in vitro.
A. Curve I *: normal hamster kidney*
Curve II *: oestrogen-treated hamster kidney*
B. Normal hamster liver
C. Curve I *: bovine serum albumin (bsa)*
Curve II *: normal hamster serum*
D. Immature rat uterus bsa standard.

Table 1. *Comparison of sucrose gradient profiles with protamine precipitation results of supernatants from tissue labelled* in vitro *with* 1 *nM* [6, 7-^3H] *oestradiol*

Tissue*	% of total supernatant radioactivity in sucrose gradient region		Precipitated by protamine	Disintegrations/min/mg of cytoplasmic protein
	8S	4S		
Independent tumour	40	>35	85 ± 11†	1896 ± 1000
Independent tumour supernatant after storage at 4°C for 7 days	40	45	87	—
RCA_1 tumour	—	—	5 ± 3	300 ± 100
Dependent primary tumour	—	>59	68 ± 16	1181 ± 336
Kidney (normal)	—	—	27 ± 27	1620 ± 200
Kidney (oestrogen-treated)	—	>80	24 ± 5	1153 ± 260
Liver ♂	—	71	18 ± 2	115 ± 13
Liver ♀	—	—	19 ± 7	375 ± 92
Uterus (rat)‡	45	>45	86 ± 3	3660 ± 2290
Liver (rat ♀)	—	90	38 ± 4	276 ± 37
Muscle (rat ♀)	—	—	16 ± 1	681 ± 188
Independent tumour labelled with [1, 2-^3H] dihydrotestosterone	14	—	31 ± 10	1505 ± 599
Serum	—	>95	5 ± 1	—
Bovine serum albumin (bsa)	—	>95	<1	—

*All are hamster tissues, unless stated otherwise.
†Means ± SD of at least three observations.
‡Uteri taken from mature rats.

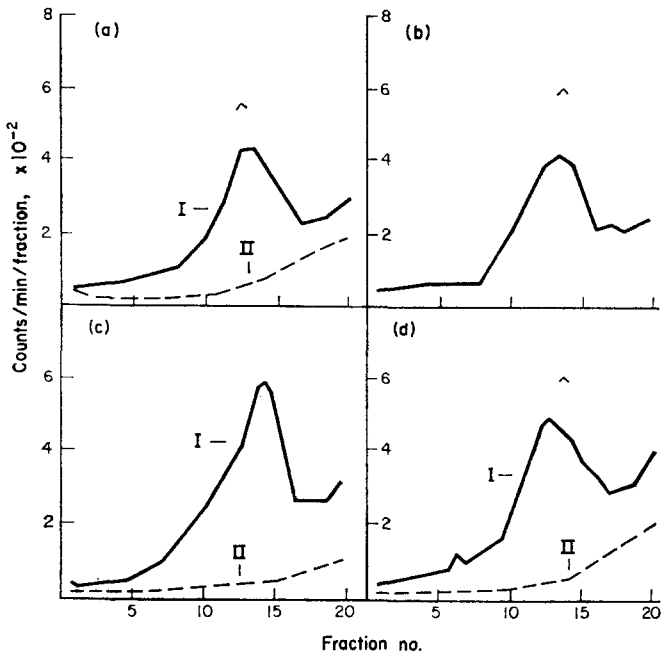

Fig. 2. Sedimentation of cytoplasmic and nuclear extracts on 5–20% sucrose gradients. Details as in Fig. 1. Nuclear extracts were obtained from tissues 0·5 hr after s.c. injection of 1 μg/[6, 7-^3H] oestradiol/100g body weight.

A. Curve I: *cytoplasmic fraction from dependent primary renal tumour (stilboestrol pellet removed 47 hr prior to excision of tumour).*
Curve II: *as for Curve* I, *but stilboestrol pellet* in situ.

B. Nuclear extract of dependent renal tumour

C. Curve I: *Dependent transplanted tumour cytoplasm (stilboestrol pellet removed)*
Curve II: *as for Curve* I, *but after protamine precipitation.*

D. Curve I: *nuclear extract from C.*
Curve II: *as for Curve* I, *but after protamine precipitation.*
Bsa standard

The profiles from the independent tumour (Fig. 3A and B) were similar to those of the dependent ones except that 8S binding was always present. This was rarely found in any of the dependent tumours growing in female hamsters. The "nuclear" extract contained only 5S binding.

Studies on the independent tumour cytoplasm

To investigate the stability of the cytoplasmic receptors, labelled cytoplasm was stored at 4°C for 7 days. Aliquots were taken on days 0 and 7, and analysed by sucrose gradient centrifugation and by protamine precipitation (Table 1). After 7 days storage the 8S peak was reduced (Fig. 3CIII). Either the 8S peak at day 0 (Fig. 3CI) was fragmented into smaller components (ca 4S) or the radioactivity was transferred to non-specific oestradiol binding proteins sedimenting in the 4S region. There was no difference in the ^3H precipitated by protamine (Table 1) which supports the former possibility. Similar results have been found for rat uterus [19, 20].

Addition of 0·3 M KCl to the gradients resulted in a loss of 8S receptor and only a single peak in the 4S region of the gradient was detected (Fig. 3BI). This suggested that the tumour 8S receptor was similar to that in immature rat uterus. In the dependent tumours where both 8 and 4S receptors were detected, the 8S receptor underwent similar salt-induced changes in sedimentation coefficient.

The effect of sulphydryl reagents was studied by incubating independent tumour tissue with or without N-ethyl-maleimide (NEM) (British Drug Houses Limited, Poole, Dorset) at room temperature for 1 hr, prior to the 2 hr incubation at 4°C with [6, 7-^3H] oestradiol. The 8S binding was completely inhibited by NEM, and the 4S region partially affected (Fig. 3DI and II). The cpm/mg of supernatant protein was also reduced from 1196 to 610, and of this protamine precipitated 86 and 33 % respectively.

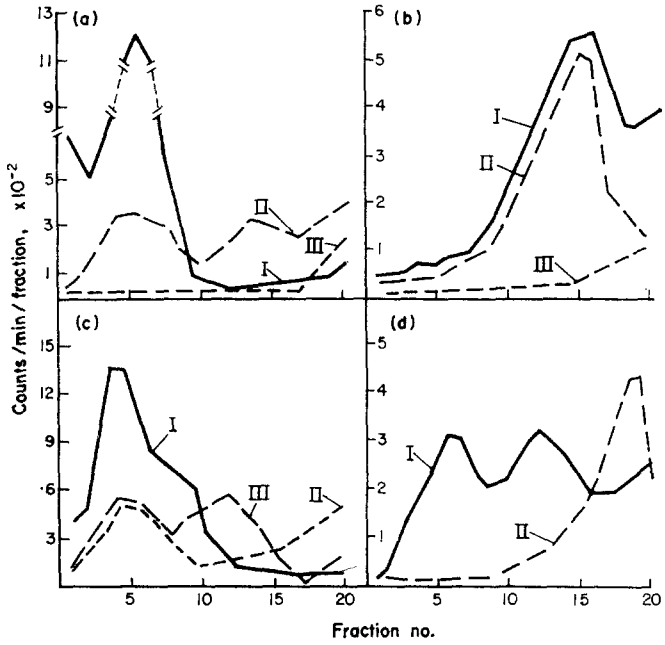

Fig. 3. Separation of cytoplasmic and nuclear extracts from independent tumours on 5–20% sucrose gradients with or without 0·3 M KCl. Intact tissue was labelled with 1 nM [6, 7–^3H] oestradiol as described in Material and Methods.

A. Curve I *and* II *: independent tumour cytoplasmic fraction*
Curve III *: as for Curve* I*, but after protamine precipitation*

B. Curve I *: independent tumour cytoplasm on a sucrose–KCl gradient.*
Curve II *: 0·3 M KCl "nuclear" extract of independent tumour as for Curve* II*, but after protamine precipitation*

C. Curve I *: independent tumour cytoplasm labelled with* [6, 7–^3H] *ostradiol* in vitro.
Curve II *: as for Curve* I*, but labelled with* [1, 2–^3H] *dihydrotestosterone.*
Curve III *: same as Curve* I*, but after storage at 4°C for 7 days.*

D. Curve I *: independent tumour supernatant*
Curve II *: as for Curve* I*, but after incubation with 10 mM N-ethyl maleimide for 1 hr before addition of* [6, 7–^3H] *oestradiol.*

These results indicate that sulphydryl groups are involved in oestradiol binding.

Because the independent tumour transplant only grows in intact but not castrate male hamsters, there seemed a possibility that the tumour might possess androgen receptors similar to those described in rat prostate [10]. To investigate this, incubations were carried out in buffer containing either 1 nM [6, 7-^{3}H] oestradiol 17β or 1 nM [1, 2-^{3}H] dihydrotestosterone, and the labelled cytoplasmic fractions analysed by sucrose gradients. The results are shown in Fig. 3C ([6, 7-^{3}H] oestradiol) and CII ([1, 2-^{3}H] testosterone). Of the three tumours examined, two gave some evidence of androgen binding in the 8S region, with no binding in the 4S area. In both these cases the amount bound was less than that of oestradiol (Table 1). When allowance is made for the lower specific activity of the [1, 2-^{3}H] testosterone (137 μc/μg as compared with 235 μc/μg for the [6, 7-^{3}H] oestradiol) it would appear that androgen receptors are present in this tumour. No studies were made on the specificity of these receptors or to identify the tritiated steroid bound under these conditions. Only oestradiol binding was detected in the nuclear extracts.

Comparison of independent tumours growing in male and female hamsters

Progesterone prevents the induction of the primary kidney tumour [7] and is probably responsible for the slower growth of the independent tumour in female as compared to male hamsters. To determine whether tumours in female hamsters were different from the comparable tumours in males, the following study

was carried out. Independent tumour tissue was transplanted into mature male and female hamsters, [6, 7-^3H] oestradiol binding was examined by sucrose gradient analysis. There was no difference in cytoplasmic or nuclear receptors between the tumours. However, tumours in males grew four times faster than those in females.

6393 *independent tumour*

This tumour grew equally well in male and female hamsters. All samples of this tumour had 8 and 4S cytoplasmic oestradiol receptors, and consequently did not differ from the other independent tumour.

Sucrose gradient analysis of the cytoplasm from the RCA_1 tumour indicated the absence of any oestradiol receptors, as did the protamine precipitation results (Table 1).

Protamine precipitations

These results are presented in Table 1 and are compared with the radioactivity associated with the 8 and 4S regions of sucrose gradients. The agreement between the amount of radioactivity associated with the 8 and 4S regions, and the amount precipitated by protamine was good for target tissues. No value can be given for normal kidney cytoplasm, because of the difficulty in assessing the difference between the 4S region, and the free radioactivity (Fig. 1AI). In liver only a small part of the 4S region was precipitated by protamine. The uptake of radioactivity by liver from female hamsters was higher than that from males. The values for liver were considerably lower than those obtained with untreated or oestrogen-treated hamster kidneys. Both hamster serum and bovine serum albumin bind oestradiol as measured by gradient analysis but negligible radioactivity was precipitated by protamine. No difference was detected between rat and hamster tissues, although rat uterine cytoplasm had the highest level of radioactivity expressed as cpm/mg of protein.

Sedimentation profiles obtained from "cytoplasmic" and "nuclear" fractions after the protamine precipitate had been removed are shown in Fig. 2CII and DII and also in Fig. 3AIII and BIII. The sedimentation profile from hamster serum was unaffected by protamine treatment.

The determination of binding constants for [6, 7-^3H] *oestradiol*

Since protamine precipitates only the radioactivity bound to specific binding sites [19], this method can be used to calculate the binding constants (Table 2). There was no difference in the dissociation constant, nor in the number of binding sites obtained for these tumours.

Differential extraction of nuclei

These experiments were carried out on nuclei from tumours that had been labelled *in vivo* with [6, 7-^{3}H] oestradiol.

(*a*) *NaCl solutions.* Nuclei from independent and dependent tumours, and oestrogen-treated kidneys were extracted with different concentrations of NaCl (Table 3). A higher proportion of radioactivity was extracted from dependent than from independent tumour nuclei. In the tumours optimal extraction occurred with 0·5 M NaCl. In the kidney 0·1 M NaCl was most effective.

(*b*) *Effect of pH.* In all instances more tritium was solubilized at pH 3 than at pH 7 or 9 (Table 4). There was no detectable difference between dependent or independent tumours or oestrogen-treated kidney nuclei. To determine whether extraction of the receptor had been achieved or whether oestradiol was being dissociated from the complex, the pH 3 and 9 extracts were chromatographed separately on Sephadex G100 columns. None of the radioactivity in the pH 3 extract was associated with the macromolecular fraction, whereas some of the pH 9 extract was. This verified that the oestradiol-receptor complex was acid labile as in other tissues [21].

(*c*) *Enzymic digestion of nuclei.* Only trypsin affected the release of tritium when compared to a control incubation without enzyme ($p < 0{\cdot}01$) (Table 5). However, the tritium released represented only a small proportion of the total nuclear content. There may be several reasons for this. Some of the radioactivity in the nuclear pellet may be associated with proteins not affected by trypsin, or possibly not enough trypsin was added. The latter is more probable, because only 30% of the total protein was degraded by the enzyme. Most of the nuclear DNA was hydrolysed by deoxyribonuclease, and the activity of the ribonuclease was checked by an incubation with yeast RNA. Neither of these enzymees caused the release of radioactivity from the nuclei. The loss of DNA during the trypsin incubation may be due to disruption of the nuclei, thus preventing them from sedimenting,

Sonication of nuclei

In an initial experiment, nuclei from an independent tumour obtained 0·5 hr after

Table 2. Binding constants for tumour cytoplasm with [6, 7-^3H] *oestradiol*

Tumour	No. estimations	Dissociation constant (moles/l. $\times 10^{-10}$)	No. of binding sites: moles/mg DNA ($\times 10^{-13}$)	No. of binding sites: moles/mg prot. ($\times 10^{-13}$)
Primary dependent tumour	1	1·3*	12·8	1·2
Independent tumour transplanted in male hamsters	3	1·0 (0·8–1·3)	16·6 (5·1–30·6)	1·9 (0·7–4·4)
Independent tumour transplanted in female hamsters	3	1·8 (1·1–2·5)	29·6 (9·7–40·3)	2·08 (0·8–3·0)

*Mean value with range and number of observations.

Table 3. NaCl extraction of nuclei from tissues obtained 0·5 *hr after* in vivo *injection of* 1 *μg* [6, 7-^3H] *oestradiol*/100*g of body weight*

Tissue	% of nuclear tritium solubilized by NaCl*: 0·1 M	0·5 M	2·0 M
Independent tumour	19 ± 10 (6)	32 ± 11 (6)	37 ± 15 (8)
Dependent primary tumour	44 ± 25 (3)	67 ± 13 (3)	34 ± 3 (3)
Kidney from oestrogen-treated hamster	58 ± 5† (3)	57 ± 2 (3)	49 ± 1 (3)

*Means ± SD, with number of observations.
†Kidney nuclei contain only 1% of the total tissue tritium whereas 40 to 60% of the tumour tritium is nuclear.

Table 4. Effect of pH on the extraction of ^3H from nuclei obtained 0·5 *hr after* in vivo *injection of* [6, 7-^3H] *oestradiol* (1 *μg*/100*g of body weight*)

Tissue	% of total nuclear ^3H extracted at pH: 3	7	9
Independent tumour	61 ± 13 (4)	44 ± 5 (3)	31 ± 16 (4)
Dependent primary tumour	53 ± 17 (3)	28 ± 12 (3)	25 ± 9 (3)
Kidney from oestrogen treated hamster	65 (1)	31 (1)	24 (1)

Results expressed as mean ± SD, number of observations.

Table 5. Effect of enzymes on the release of tritium from independent tumour nuclei obtained 0·5 *hr after a s.c. injection of* [6, 7-^3H] *oestradiol* (1 *μg*/100*g of body weight*)

Enzyme	% of total nuclear tritium remaining with the nuclei: without enzyme	with enzyme	% of untreated controls: Protein	DNA
Trypsin	88 ± 7	61 ± 15	66 ± 16	60 ± 10
Deoxyribonuclease	93 ± 2	93 ± 2	102 ± 19	3 ± 5
Ribonuclease	93 ± 2	93 ± 3	110 ± 14	104 ± 7

Results are expressed as means ± SD, 5 observations remaining in the control incubation.

Table 6. Amount of oestradiol in independent tumour nuclei, 0·5 hr after the s.c. injection of [6, 7-^3H] oestradiol-17β (1 μg/100g of body weight)

Pretreatment	None	Oestradiol-17β*	Oestradiol-17α	U11100A	Stilboestrol	Stilboestrol† implant
Oestradiol content (moles × 10^{-17}/μg DNA)	47 ± 25‡ (13)	11 ± 6 (6)	53 ± 35 (6)	7 ± 3 (4)	7 ± 4 (5)	11 ± 9 (4)

*Compounds were administered as a single s.c. injection (10 μg/100 g body weight) 0·5 hr prior to the injection of the [6, 7-^{3}H] oestradiol.
†Tumours were taken from animals with the stilboestrol pellet *in situ*.
‡Means ± SD (with number of samples).

in vivo injection of [6, 7-^3H] oestradiol were sonicated for either 30, 150, 300 or 600 sec with 60 sec cooling in an ice–salt mixture between each 30 sec sonication. The suspensions were centrifuged for 1 hr at 10^5 **g**, and the radioactivity determined in both supernatant and pellet fractions. After 30 sec sonication, 10·2% of the nuclear radioactivity was solubilized, and 18·5% after 600 sec. In subsequent experiments, nuclei were sonicated for 30 sec only, and centrifuged differentially. Some radioactivity (25 ± 8%) sedimented with the 100 g pellet, as did 34 ± 8% of the total DNA. The chromatin fraction sedimented at 10^5 **g** for 1 hr contained most of the DNA (64 ± 8%), but only the same amount of radioactivity (28 ± 5%) as the soluble fraction.

Effect of inhibitors on the association of oestradiol with the nuclear chromatin of independent tumours

To determine the specificity of oestradiol binding to the nuclei, hamsters were injected with 10 μg of an oestrogen or an oestrogen analogue 30 min before the injection of 1 μg [6, 7-^{3}H] oestradiol/100 g body weight. After a further 30 min, the animals were killed and nuclei isolated. The amount of radioactivity associated with the nuclei is given in Table 6. Oestradiol-17β stilboestrol and U11100A all significantly reduced the amount of oestradiol/μg DNA in tumour nuclei ($p < 0{\cdot}01$). Oestradiol-17α had no effect, indicating that the binding sites have oestrogen specificity. Insufficient dependent tumours were available for a comparable study.

The RCA_1 tumour had such a low oestradiol/μg DNA value (5 ± 3) that inhibition studies were not possible.

Normal hamster uterus

In order to facilitate comparison of these results with those obtained with a normal oestrogen-sensitive hamster tissue, some experiments were also carried out with hamster uterus. In immature or ovariectomized, mature animals only 8S cytoplasmic receptor was detectable but in cycling animals the type of receptor varied. On days 1 and 2 (day 1 = post oestrus discharge) only 4S receptor was present whilst on days 3 and 4 both 8 and 4S were present. The nuclear 5S receptor did not vary with the cycle. The cytoplasmic receptor had a $K_D = 9 \times 10^{-11}$ M at 4°C and the number of cytoplasmic binding sites was 3×10^{-13} mole/mg supernatant protein throughout the cycle. The number of binding sites per cell varied, values of 71×10^{-13} mole/mg DNA being obtained on day 2 and 47×10^{-13} mole/mg DNA on day 4.

DISCUSSION

The observation that dependent primary, dependent transplant and independent transplant tumours all possess oestrogen receptors is interesting. In all types of tumour, a single nuclear 5S receptor was detected. The dependent tumour cytoplasm always contained either 4 or 8+4S receptors and, by analogy with the situation existing in the mature rat uterus [22], it is probable that the oestrogen pellet *in situ* is responsible for the formation of the 4S receptor. 4S cytoplasmic receptors have been described for other tissues [19, 20, 23]. In independent tumour cytoplasm either 8 or 8+4S receptors were detected. However, only part of the tritium in the 4S region is attached to specific proteins as this region of the gradient is only partly affected by protamine and N-ethylmaleimide [24]. The 8S receptor was lost after treatment with either of these reagents.

Additional evidence for the presence of specific 4S binding was obtained from the "ageing" cytoplasm experiment. Increased protamine-precipitable 4S binding was detected

after storage at 4°C, indicating that it was not due to albumins. Vonderhaar *et al.* [20] have described a similar "ageing" effect using rat uterine cytoplasm. It is probable that there is a relationship between the tumour 8 and 4S receptors similar to that existing in native rat uterus [19].

Due to the small number of dependent tumours available, most studies on the nuclear binding of oestradiol were confined to independent tumours. In both types of tumour the association of oestradiol with the nuclear receptor was acid-labile. Previously, Noteboom and Gorski [25] had shown that the release of oestradiol from rat uterine nuclei at acid pH was not due to an activation of cathepsins. Some of the nuclear oestradiol was extractable with 0·5 M NaCl suggesting an association with chromatin, but the proportion extracted was much lower than with mammary tumours [9].

In independent tumour nuclei, oestradiol was associated with protein, as only trypsin effected the release of radioactivity. Sonication studies gave results that were consistent with a chromatin localization, but the results were less clear-cut than with mammary tumours [21].

In vivo pretreatment of hamsters with stilboestrol, oestradiol-17β or U11100A, but not oestradiol-17α, significantly reduced the amount of oestradiol associated with nuclei from independent tumours. This suggests that the receptor association was related to the biological potency of the oestrogen. Other competition studies using *in vivo* or *in vitro* pretreatment of uterine tissue [25–27] have obtained similar results.

Only one comparable study on receptor specificity was carried out on tumour cytoplasm. The receptor from independent tumour cytoplams was shown to have little specificity for dihydrotestosterone. Where 8S binding was observed, it was not precipitable by protamine, suggesting that the dihydrotestosterone was not bound to the same molecule as oestradiol. Mainwaring [10] found dihydrotestosterone-specific, but not oestradiol-specific binding in rat prostate cytoplasm. Thus, the two receptors have different specificities even though they have similar sedimentation constants.

The protamine studies provide further evidence for the presence of specific oestradiol binding in the tumours. The cytoplasmic 8 and 4S, and the nuclear 5S binding were all precipitable by this reagent. This is similar to the situation existing in rat uterus and DMBA tumour [5]. The amount of radioactivity precipitated from liver and muscle was insignificant. However, both oestrogen-treated and untreated hamster kidneys contained appreciable quantities of precipitable binding. No explanation can be offered for this.

The dissociation constants for the dependent and independent tumour cytoplasmic receptors were the same as those determined for rat uterus [19]. The accumulated evidence would suggest that the hamster tumours possess the same or similar oestradiol receptors to those described for other tissues.

On the whole the results do not demonstrate acorrelation between the hormone dependence (or responsiveness) of the tumours and their oestradiol binding potency. All the tumours studied possessed oestradiol receptors, but this is not necessarily proof of oestrogen dependence. The independent tumour *in vivo* retains less oestradiol than the dependent tumour [6], but *in vitro* the tumours are similar. Probably the independent tumour is also oestrogen-dependent [8], but its oestrogen requirement is much less than that of the primary tumour as the latter is dependent on an exogenous oestrogen source. The mechanism by which this difference evolves is unkown. If there was a gradual reduction in the ability of the tumour nuclei to accept oestradiol from the cytoplasmic receptor, then with successive transplantations, the tumour may develop the ability to survive on decreasing levels of oestrogen. Eventually, the tumour may exist and maintain growth on the endogenous levels of oestradiol in the male hamster. These experiments do indicate that tumours exist which contain cytoplasmic receptors which may not have any functional significance. The results are compatible with this view.

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The Relationship between the 'Cell Loss Factor' and the Immediate Response to Radiation in Animal Tumours

J. DENEKAMP

The Gray Laboratory, C.R.C. Research Unit in Radiobiology, Mount Vernon Hospital, Northwood, Middlesex, England

Abstract—*An analysis of the cellular proliferation kinetics of* 10 *more tumours confirms the earlier finding that all carcinomas have high cell loss factors(ϕ) whilst, in general, the sarcomas have lower values. The clear distinction between sarcomas and carcinomas is less pronounced in this series, with some significantly higher values of ϕ in the slowly growing sarcomas. The radiation response was very different in the two groups of tumours: the sarcomas all continued to grow for several days after* 1–2 *krad before exhibiting a shrinkage in volume; the carcinomas, on the other hand, shrank rapidly after irradiation. It is postulated that the initial shrinkage in the carcinomas is not a direct result of radiation-induced cell death, but is rather an unmasking of the normal rate of cell loss when cell production is halted by a radiation-induced mitotic delay. The absence of such immediate shrinkage in the relatively slowly growing sarcomas may be due to differences in the vascular architecture, the mode of death after irradiation or the relative amount of oedema resulting from radiation injury.*

INTRODUCTION

IN RECENT years a number of publications have appeared on detailed analyses of the proliferation kinetics of normal and malignant tissues *in vivo* [e.g. 1–3]. It is hoped that ultimately this information will be of use in improving the treatment of human malignancies. As yet there are still relatively few studies of the relationship between the radiation response and the proliferation kinetics of the same tissues.

In a previous publication [2] on 3 rat sarcomas and 1 mouse carcinoma, the author showed that the overall growth rate of the sarcomas was due mainly to variations in the cell cycle time, with only small differences in the growth fraction and cell loss factor. In the relatively slowly growing carcinoma the mean cell cycle time was short, and the slow overall growth of the tumour was attributable only to extensive cell loss from the tumour volume. A review of other published data [2] showed these relationships to be more general: all sarcomas seemed to have growth rates determined largely by their cell cycle times, whereas all the carcinomas had fairly short cycle times but large cell loss factors varying from 0·70–0·95.

During the study of the carcinoma and the three sarcomas it was also noted that there was a marked difference in their regression rates after a single dose of 1–2 krads of X-rays: the sarcomas continued to increase in volume for several days after irradiation whereas the carcinoma regressed immediately, losing up to 20% of its volume within 24 hr [4]. It seemed likely that these differences in initial regression rates might be correlated with the cell loss

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factor measured in unirradiated tumours. Consequently, the investigation was extended to 10 more tumours of varying growth rates to determine the relationship, if any, between the initial radiation response and the cell loss factor.

MATERIAL AND METHODS

The animals used were John's strain Wistar rats and C_3H and BALB/C mice. First transplants from a spontaneous tumour and established line transplanted tumours in the 17th to 500th generation of transplantation were used. The tumours chosen varied in growth rate, number of transplants since they first arose, tissue of origin, and degree of differentiation. The methods by which the rat tumours were implanted have been described in detail in a previous publication [2]. The mouse tumours were all implanted as pellets of minced tumour tissue encapsulated in calcium alginate to reduce metastasis and infiltration into surrounding tissues. They were implanted subcutaneously in 6 to 8-week old male recipients and were observed until the tumours reached 2 to 3 mm mean dia. Beyond this size the three perpendicular diameters were measured 3 to 5 times weekly using vernier calipers. Groups of at least 6 tumours were measured in this way to obtain growth curves extending to 15 or 20 mm mean dia. A further group of 5 to 8 animals had their tumours irradiated with a large single dose of 1000 to 2000 rad of 250 kVp X-rays, when they were between 7·5 and 10 mm mean dia. The methods of irradiation have been described in detail by Thomlinson [5] for the rats and by Howes [6] for the mice. Animals anaesthetised with amytal or nembutal were irradiated with a collimated beam, approximately 2·0 to 2·5 cm dia. The irradiated tumours were measured at least 5 times per week to determine the change in the growth curves induced by irradiation. The growth measurements were analysed by a simple computer programme; geometric means were taken of the three diameters for each tumour, and the mean diameters for each batch of tumours was averaged for each day, taking the day on which they exceed 8 mm mean diameter or are irradiated as zero time.

The complete cell kinetic analysis of 4 of the tumours—RIB_5, SSO and SSB_1 rat sarcomas and the C_3H carcinoma—has been published previously [2]. In order to obtain the cell loss factor, for the additional 10 tumours, the volume doubling time (T_D), the initial labelling index (LI), and the duration of DNA synthesis (T_S), were obtained as part of a more complete analysis [Denekamp and Kallman, in preparation]. The volume doubling times were estimated from the growth curves. For autoradiography, animals were injected with tritiated thymidine (0·5 to 2·0 μCi/g body weight, 360 mCi/mM) when their tumours were in the range 7·5 to 10 mm mean dia. After sacrifice at intervals from 1 to 20 hr after injection, their tumours were removed, bisected and fixed in 10% formol alcohol or 5% formol saline for 48 hr. The tumours were processed and sectioned at 4 μm thickness. The slides were then dipped in Ilford K5 or Kodak NTB2 nuclear emulsion, exposed for 2 to 6 weeks, developed, and stained with haemotoxylin and eosin. At least 2000 cells were scored for the labelling index in tumours sampled 1 hr after injection. All sections were scored for the percentage of labelled mitoses, between 100 and 200 mitoses being counted at each point. A cell was considered labelled if there were more than three grains above the background level.

The potential doubling time for each tumour was calculated from these autoradiographic data using the equation [7]

$$\text{Potential doubling time, } T = \frac{\lambda T_s}{LI}, \qquad (1)$$

where λ is a correction factor for the non-linear distribution of cells through the cell cycle. λ is obtained from the equation

$$\lambda = \frac{T}{T_s}\left\{\exp\left[\frac{\ln 2(T_{G_2}+T_s)}{T}\right] - \exp\left[\frac{\ln 2\, T_{G_2}}{T}\right]\right\} \qquad (2)$$

by an iterative procedure. The potential doubling time is strictly a prediction of the time it will take for the cell number to double, assuming no loss of cells and allowing for the fact that not all cells are in the division cycle. This doubling time will equal the volume doubling time of the tumour if the mean cell volume remains constant and if the proportion of stroma to each parenchymal cell does not change. If a discrepancy between the two exists, this can be interpreted as cell loss if the above conditions hold true [7]. A cell loss factor, ϕ, can be calculated from

$$\phi = 1 - \frac{T}{Td} \qquad (3)$$

RESULTS

(a) Radiation response

Figure 1 shows control growth curves for the different carcinomas, each point representing the mean diameters of at least 6 tumours. For each curve the average volume doubling time was determined over the size range used for the kinetic and radiation experiments, i.e. 7·5 to 10 mm (Table 1). All the tumours exhibited a slowing of the growth rate as they increased in size. Figure 2 shows control growth curves for the sarcomas, from which the volume doubling times shown in Table 2 were derived. A wide range of control growth rates was exhibited in the different tumours (Tables 1 and 2) but in general the carcinomas had longer doubling times than did the sarcomas.

Figure 3 and 4 show the radiation response of the tumours, each point representing the mean diameter of at least 5 mice. The variation of individual tumours, within any tumour type, was remarkably small in the first few days after irradiation, but this variation increased with time, particularly during the phase of regrowth.

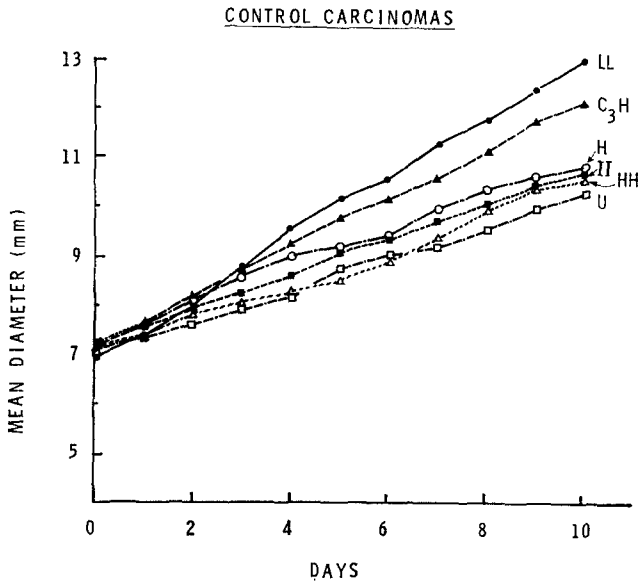

Fig. 1. The growth of unirradiated carcinomas. Each point represents the mean diameter of a batch of at least 6 animals.

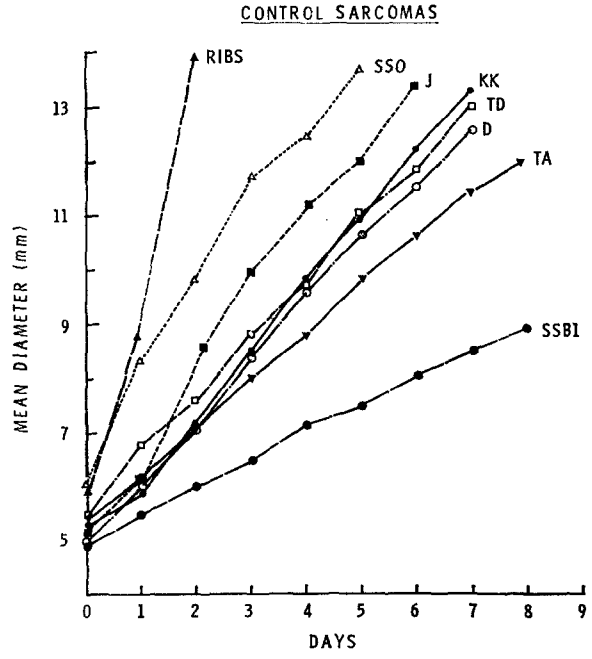

Fig. 2. The growth of unirradiated sarcomas. Each point represents the mean diameter of a batch of at least 6 animals.

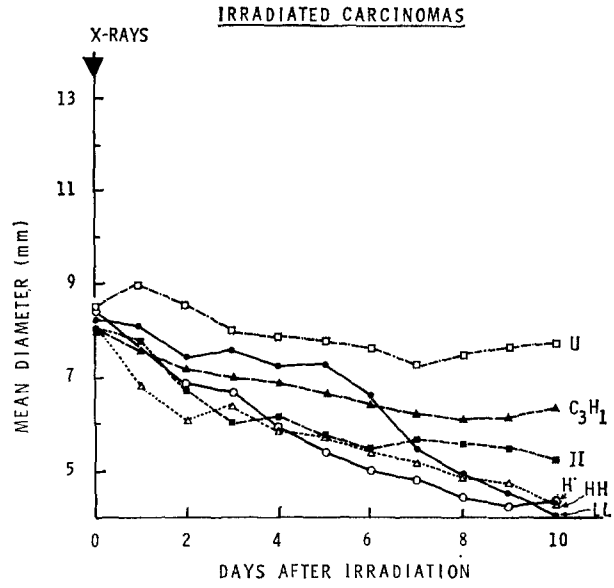

Fig. 3. The growth of carcinomas after irradiation with 1500 to 2000 rad of X-rays. Each point represents the mean diameter of at least 5 animals. Five of the six groups of tumours show a reduction in volume within 24 hr of irradiation.

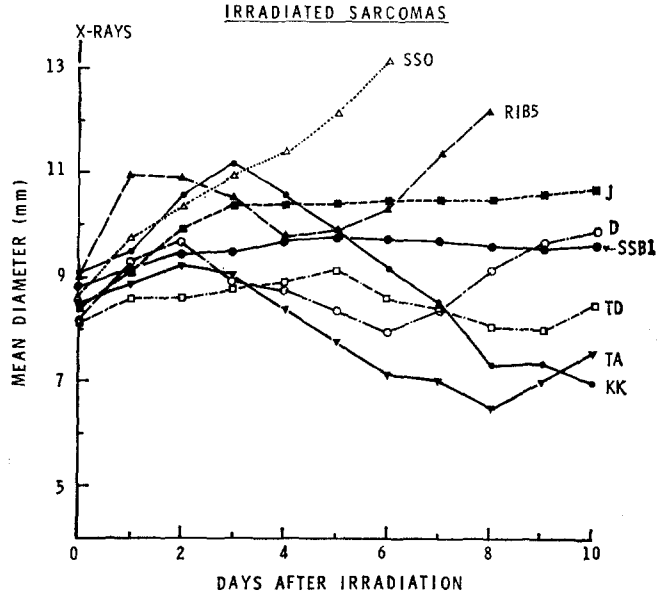

Fig. 4. The growth of sarcomas after irradiation with 1000 to 2000 rad of X-rays. Each point represents the mean diameter of at least 5 animals. None of the groups of tumours shows a decrease in volume until 3 days after irradiation.

Table 1. *Carcinomas*

Tumour	Animal	*Origin	Histology	†Degree of differentiation	Transplant generation	T_D (*hr*)	T (*hr*)	LI (%)	T_s (*hr*)	ϕ	Dose of radiation (rads)	Volume change 24 hr after irrad. (%)
C3H1	C3H	S. mammary	Adenocarcinoma	+ +	1	108	33	16	7	0·70	1500	−11
KHH	C3H	S. mammary	Adenocarcinoma	—	73–73	122	32	22	8	0·74	2000	−17
KHU	C3H	S. mammary	Carcinoma	− −	92–96	140	31	18	7	0·78	2000	+15
KHHH	C3H	S. mammary	Adenocarcinoma	+ +	17–19	132	43	16	9	0·67	2000	−46
KHII	C3H	S. mammary	Adenocarcinoma	+	17–21	120	63	12	10	0·48	2000	−17
KHLL	C3H	S. salivary gland	Carcinoma	− −	26–29	62	22	34	8	0·65	2000	−6

*S. =spontaneous.
†− − = anaplastic; — = undifferentiated; + = poorly differentiated; + + = well differentiated.

Table 2. *Sarcomas*

Tumour	Animal	*Origin	Histology	†Degree of differentiation	Transplant generation	T_D (*hr*)	T (*hr*)	LI (%)	T_s (*hr*)	ϕ	Dose of radiation (rads)	Volume change 24 hr after irrad. (%)
RIB5	Rat	Benzopyrene I	Fibrosarcoma	− −	450–550	24	24	30	9	0·0	1500	+106
SSO	Rat	S. orbit	Fibrosarcoma	+	30–40	34	36	16	8	0·0	1000	+48
SSB_1	Rat	S. mammary	Fibrosarcoma	+ +	25–35	78	58	13	10	0·25	1500	+23
KHD	C3H	Methylcholanthrene I	Sarcoma	+	164–169	43	17	44	8	0·60	2000	+51
KHJ	C3H	? radiation I	Sarcoma	+	142–148	29	28	31	10	0·04	2000	+34
KHTA	C3H	? Ear tag I	Fibrosarcoma	−	131–145	48	44	21	11	0·08	2000	+17
KHTD	C3H	from KHTA	Sarcoma	− −	127–133	48	28	30	10	0·41	2000	+19
KHKK	BALB/C	S. muscle	Rhabdomyosarcoma	− −	23–28	39	12	67	9	0·68	2000	+18

*I = induced; S. = spontaneous.
†− − = anaplastic; — = undifferentiated; + = poorly differentiated; + + = well differentiated.

It is apparent from Fig. 3 that of the 6 types of carcinoma that were irradiated, all showed a rapid response to irradiation. Five of the curves show regression within 24 hr of irradiation and the sixth, KHU, only enlarged slightly for 1 day before shrinking. In the sarcomas, however, the response to irradiation was always later. They all continued to grow for 2 to 3 days before regressing after large doses and three of the sarcomas did not shrink at all, but merely grew more slowly, or not at all for some time. The change in volume in the first 24 hr is shown in Tables 1 and 2.

The percentage of labelled mitoses was determined over the first 20 hr after injection of 3HTdR for each tumour type and from these data the duration of DNA synthesis was estimated. These estimates of T_s and of the labelling index are shown in Tables 1 and 2, together with calculated values of the potential doubling time. It can be seen in Table 2 that the potential doubling times of the sarcomas are in most cases similar to the measured volume doubling times yielding small or zero cell loss factors. In the carcinomas however, there is a large discrepancy in every instance. Since there is no noticeable alteration in mean cell volume or tumour architecture over the 7·5 to 10 mm size range used, the discrepancy can be attributed to the loss of cells from the tumour volume. Values of ϕ for the carcinomas range from 0·48 to 0·78; the values for the sarcomas are lower: five of the tumours have cell loss factors of less than 0·25.

DISCUSSION

In this study, as in those reviewed previously [2], all the carcinomas exhibit a high cell loss factor, whereas the sarcomas have lower values. However, three of the sarcomas studied here, KHD, KHTD and KHKK, show a significantly high value of ϕ. None of the sarcomas shows any sign of shrinkage for at least 2 days after a large single dose of radiation, whereas all the carcinomas exhibit rapid and extensive shrinkage, often losing 10 to 20% of their volume within 24 hr. The doses of radiation used in these experiments would be expected from *in vitro* data to produce a significant period of mitotic delay during which the cells accumulate in the premitotic G_2 phase [8, 9]. At the end of the mitotic delay the cells would progress through mitosis. Many of the radiation damaged cells, failing to complete division successfully, would die at this stage. After doses of 1 to 2 krads the mitotic delay would be equivalent to 1 or 2 cell cycle times. Therefore little radiation-induced death should be manifested within the 24-hr period unless interphase death occurs. Although mitotic delay results in a cessation of cell production, continued accumulation of cells in the later stages of the cell cycle would result in an increase in the mean cell volume. With cell production halted in this way the cell loss, which is normally balanced by production, could manifest itself as a reduction in the tumour volume. This is consistent with the fact that the carcinomas exhibiting high cell loss factors also have a rapid post-irradiation shrinkage, whereas the sarcomas which have lower cell loss factors all continue to grow. However, the correlation between shrinkage and ϕ is not a good one, since the three sarcomas with cell loss factors in excess of 0·40 also do not shrink for several days after irradiation (Tables 1 and 2). Thus the correlation between the time of shrinkage and the histological type is better than the correlation with values of ϕ determined here.

When cells in a tumour die they can be removed from the tumour volume by autolysis or phagocytosis or they can remain to form a necrotic mass. It is possible that the difference in the removal rate correlates with the vascular architecture within the tumour and the position of any dead cell relative to the surrounding blood vessels. Most carcinomas have a well defined vascular architecture which can be easily identified in haematoxylin and eosin stained sections. The blood vessels, with supporting stromal elements, often supply cords or lobules of tumour cells. The labelling index sometimes varies across the lobule or cord, being high adjacent to the blood vessel and falling off with distance. Occasionally, the centre of such lobules is a confluent mass of necrotic cells, presumably due to death caused by starvation as the cells are pushed away from their source of nutrients. Such structures have been described in detail by Thomlinson and Gray [10] and Tannock [11]. In sarcomas, however, the blood vessels are often difficult to identify, possibly because the fibrous components of the stroma are not so recognisably different from fibrous tumour cells. It may also be that the blood volume/tumour cell ratio in sarcomas is smaller, since Gullino [12] found 4 to 12% of tumour volume occupied by blood in several carcinomas but only 1% of the volume in the one sarcoma he studied. Some sarcomas have non-cellular differentiated elements that remain after the cells die from irradiation, but the lack of immediate regression is common to all sarcomas, both very differentiated, e.g. SSB_1, and very anaplastic, e.g. RIB_5.

If the difference between sarcomas and carcinomas is not related to the vascular supply, perhaps there is a difference in the amount of oedema following radiation damage. Alternatively, there is some evidence to suggest that somatic cells *in vivo* do not only die in mitosis. Hug, Tannock and Mendelsohn (discussion at Williamsburg meeting 1970) have each observed pyknotic cells in carcinomas within several hours of receiving doses of between 300 and 700 rads. It is possible that these early pyknotic cells are a result of interphase death, and the relative importance of interphase and mitotic death may vary in the different histological types of tumour. Such a variation, if associated with a better organized system for normal removal of dead cells in the carcinomas, could account for the different patterns of shrinkage after irradiation.

The cause of the different responses to radiation is of more than just academic interest. The rapidly shrinking C_3H carcinoma shows a very rapid and extensive re-oxygenation of its radioresistant hypoxic cells after a single dose of radiation [6]. The sarcoma RIB_5, which continues to enlarge after irradiation, also re-oxygenates rapidly, but the hypoxic proportion does not fall to a lower level than that which exists before irradiation. Re-oxygenation is caused by an improvement in the oxygen concentration in the vicinity of the hypoxic tumour cells, and its time course and extent could well be governed by the rate of removal of radiation-killed cells which lie between the source of oxygen and the hypoxic

cells. In tumours which shrink rapidly, this removal of cells must be an effective process. If it could be predicted which tumours re-oxygenate well, costly procedures such as high pressure oxygen or neutrons, which are being considered for improving radiotherapy of human tumours, could be reserved for the non-re-oxygenating tumours which might be expected to benefit most.

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Benzoylhydrazine Carcinogenesis in Lungs and Lymphoreticular Tissues of Swiss Mice

BELA TOTH

The Eppley Institute for Research in Cancer, University of Nebraska College of Medicine, 42nd and Dewey Avenue, Omaha, Nebraska 68105, *U.S.A.*

Abstract—*Administration of* 0·01% *benzoylhydrazine in the drinking water of* 6 *week old randomly bred Swiss mice for the remainder of their lifetime enhanced the development of lung tumors and malignant lymphomas. The incidence of lung tumors rose from* 12 *to* 68% *in the females and from* 10 *to* 63% *in the males, while the incidence of malignant lymphomas increased from* 14 *to* 36% *in the females and from* 1 *to* 10% *in the males, as compared with the controls.*

The possible mechanism of action of substituted hydrazines in chemical carcinogenesis is discussed in the light of the obtained results.

INTRODUCTION

AFTER the demonstration that hydrazine, one of the metabolites of isonicotinic acid hydrazide (INH) is carcinogenic [1, 2], attention has been directed towards two main problems. Firstly, several investigators studied the other metabolites of INH, isonicotinic acid and 1-acetyl, 2-isonicotinoylhydrazine, for tumor producing capability [3–5]. Secondly, it was believed to be promising to look for substituted hydrazines as possible carcinogens, which are widely distributed in the environment and used in industry, agriculture and even in medicine. Several of these compounds such as the methyl-, dimethyl-, diethyl-, and phenylhydrazines were studied for neoplastic actions in mice, hamsters, and rats, and the findings clearly showed their capacity to evoke tumors of lungs, liver, intestines, etc. [2, 6, 7–10]. As a matter of fact, benzoylhydrazine, when given daily by stomach tube feeding [6], has been found to be carcinogenic to the lungs of Balb/c/Cb/Se mice. In another study, however, virgin Swiss female mice received benzoylhydrazine by gavage on 5 days each week for 50 weeks without tumor producing effect [2].

In this laboratory, systematic investigations on the relationship between the chemical structures of hydrazine derivatives and tumor development at specific organ sites in Swiss mice and Syrian golden hamsters was initiated in 1968. In Swiss mice hydrazine in sulfate form induced lung tumors; symmetrical dimethylhydrazine di HCl evoked tumors of blood vessels and lungs [11, 12].

The present report records the carcinogenic response of Swiss mice to another chemical of this series to the oral lifelong administration of benzoylhydrazine.

MATERIAL AND METHODS

Swiss albino mice from the colony randomly bred by us since 1951 were used. They were housed in plastic cages with granular cellulose bedding, separated according to sex in groups of ten, and given Rockland diet in pellets (A. E. Staley Mfg. Co., Decatur, Illinois) and tapwater with the chemical in it *ad libitum* as described below.

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The chemical used was benzoylhydrazine (BH), K and K Laboratories, Inc., Plainview, New York.

Toxicity study was carried out prior to the chronic experiment. Five dose levels of benzoylhydrazine such as 1, 0·1, 0·01, 0·001 and 0·0001 % were administered in the drinking water for 35 days to Swiss mice. By taking into account four parameters, survival rates, body weights, chemical consumption figures and histological changes, the 0·01 % was found to be suitable for the lifelong treatment. The description of this method has been submitted for publication [13].

The solutions were prepared thrice weekly, and the total consumption of water containing BH was measured at the same intervals during the treatment period. The solution was contained in brown bottles because of the possible light sensitivity of the chemical. The experimental group and the treatment were as follows.

BH was dissolved in the drinking water as 0·01 % solution and was given for the life of 50 female and 50 male mice which were 6 weeks old at the beginning of the experiment. The average daily water consumption containing BH per animal was 5·7 ml for the females and 8·5 ml for the males. Therefore, the average daily intake of BH was 0·57 mg for a female and 0·85 mg for a male.

The experimental animals were carefully checked and weighed at weekly intervals and the gross pathologic changes were recorded. The animals were either allowed to die or were killed with ether when found in poor condition. Complete necropsies were performed on all animals. All organs were examined macroscopically and were fixed in 10 % buffered formalin. Histological studies were done on liver, kidney, spleen, four lobes of lungs as well as on those organs which showed gross pathological changes. Sections from these tissues were stained routinely with hematoxylin and eosin and with additional special methods when necessary.

RESULTS

The survival rate at 10-week intervals is recorded in Table 1. It appears from this data that BH treatment reduced somewhat the survival when compared with the corresponding controls [16]. The average weekly weight curves of the treated and control mice were recorded throughout the experiment and no significant difference was noted between the two groups.

Table 2 presents the number and incidences of various types of tumors with their latent periods. As can be seen two main types of neoplastic lesions were observed.

Lung tumors

In the BH-treated animals, 34 females developed 124 lung tumors with an incidence of 68 %. Out of these, 18 mice had 60 adenomas and 16 had 22 adenocarcinomas and 42 adenomas. The average latent period for these lung tumors was 88 weeks, the first was found at the 64th week and the last at the 113th week. In BH-treated males, 31 mice developed 111 lung tumors with an incidence of 63 %. Out of these, 18 had 55 adenomas, 11 had 19 adenocarcinomas and 35 adenomas and 2 had 2 adenocarcinomas. The average latent period for these lung tumors was 77 weeks, the first was observed at 50 weeks and the last at the 97th week.

Table 1. *Treatment and survival rate in benzoylhydrazine (BH)-treated and control swiss mice*

Group	Treatment	Initial No. and sex of mice	No. of survivors (age in weeks)												
			10	20	30	40	50	60	70	80	90	100	110	120	130
1	0·01% BH in drinking water daily for life	50♀	50	49	49	47	47	47	41	31	17	8	2	—	
		50♂	49	48	43	42	42	34	25	16	2	—			
2	Control	110♀	109	109	107	104	96	89	73	57	41	23	11	1	—
		110♂	110	95	91	86	67	55	41	22	6	1	1	—	

Table 2. Treatment and tumor distribution in benzoylhydrazine (BH)—treated and control Swiss mice

Group	Treatment	Effective No. and sex of mice	Animals with							
			Lung tumours			Mal. lymphomas			Other tumors	
			No.	%	Latent Period*	No.	%	Latent Period*		Latent period*
1	0·01% BH in drinking water daily for life	50♀	34	68	88 (64–113)	18	36	87 (31–113)	2 Adenocarcinomas of breast (70, 103) 1 Adenoma of glandular stomach (82) 1 Adenocarcinoma of ovary (89) 1 Papilloma of skin (82)	1 Adenoma of kidney (91) 1 Angioma of ovary (94) 1 Angioma of liver (82)
		49♂	31	63	77 (50–97)	5	10	78 (71–84)	3 Adenocarcinomas of glandular stomach (73, 75, 83) 2 Granulocytic leukemias (38, 59) 2 Adenomas of glandular stomach (50, 53)	1 Polypoid adenoma of caecum (59) 1 Sarcoma of bladder (97) 1 Papilloma of forestomach (86)
2	Control	110♀	14	12	90 (64–119)	16	14	77 (39–115)	9 Adenocarcinomas of breast (46, 60, 64, 68, 73, 91, 93, 98, 110) 3 Subcutaneous sarcomas (68, 82, 82) 3 Hemangiomas of liver (69, 77, 84) 1 Sex cord mesenchyma tumor (99) 1 Papilloma of forestomach (112)	1 Luteoma (99) 1 Subcutaneous fibroma (87) 1 Granulosa cell tumor (65) 1 Malignant plasmacytoma (71) 1 Hemangioma of ovary (42)
		110♂	11	10	74 (47–110)	2	1	77 (73–82)	2 Hemangiomas of liver (72, 80)	

* Age in weeks.

Grossly and histologically these lesions were similar to those described in earlier publications in this laboratory [14].

Malignant lymphomas

In the BH-treated females, 18 animals developed this neoplasm with an incidence of 36%. Out of these, 1 was a stem-cell (undifferentiated) type, 1 was a lymphocytic type, 12 were histocytic types, 2 were mixed (lymphocytic and histiocytic) cells and 2 were unclassifiable because of advanced decomposition. The average latent period for the lymphoreticular tumors was 87 weeks, the first was found at the 31st week and the last at the 113th week. In the treated males, 5 mice developed malignant lymphomas with an incidence of 10%. Out of these, 4 were diagnosed as histiocytic types and 1 as unclassifiable. Their average latent period was 78 weeks, the first was observed at the 71st week and the last at the 84th week.

Macroscopically and cytologically the observed lymphoreticular tumors were identical to those published earlier in this mouse strain [15].

Other tumors

As can be seen in Table 2, few other types of tumors were seen in the various groups. Because they appeared in low incidences, they cannot be related to the treatment.

The detailed description of tumors in the control animals is given in an earlier publication [16]; nevertheless, they are included in Table 2 for comparative purposes.

DISCUSSION

The present findings demonstrate that the continuous treatment of 0·01% BH solution in the drinking water for life induced tumors in the lungs and lymphoreticular system in randomly bred Swiss mice. The incidence of lung tumors rose from 12–68% in the females and from 10–63% in the males, while the incidence of malignant lymphomas increased from 14–36% in the females and from 1–10% in the males, as compared with the controls.

The current observation concerning the induction of lung tumors in mice is in agreement with the finding of a previous study in another laboratory which showed the lung tumor inducing capability of BH when given daily by stomach tube feeding to Balb/c/Cb/Se mice [6]. In addition, the treatment significantly elicited the development of malignant lymphomas which has not been seen in the earlier study.

The investigation is part of a systematic study on the possible relationship between the chemical structure of hydrazine derivatives and tumor development at specific organ sites. By using identical methods of treatment, i.e. oral *ad libitum* drinking water administration and the same host, Swiss mice, hydrazine significantly enhanced the appearance of lung tumors [16], while the symmetrical dimethylhydrazine evoked lung tumors and in addition induced multiple angiosarcomas in the blood vessels [11, 12]. Thus, the obtained results with benzoylhydrazine here provides another notable example—the induction of lung and lymphoreticular tumors in this series of experiments.

With regard to the role of action of benzoylhydrazine in carcinogenesis the following may be kept in mind. Even though the metabolism of the compound so far has not been investigated, it could very well be that the ring structure might be responsible for the induction of malignant lymphomas and the hydrazine portion for the lung tumorigenesis. This possible explanation is supported by those studies which showed the lung tumor inducing capability of oral hydrazine administration in mice [16] and by implications of the findings that in man the contact with benzene is associated with the appearance of various sorts of hematologic abnormalities and leukemias, even though to this day no one has satisfactorily proved that benzene can cause leukemia in experimental animals [17–20].

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The Effect of Heparin on the Initial Phase of Metastasis Formation

P. HILGARD, L. BEYERLE,* R. HOHAGE, V. HIEMEYER and M. KÜBLER

Section of Coagulation Research, Center of Internal Medicine and Pediatrics, University of Ulm, Ulm, Germany.

Abstract—*Circulating tumour cells in the blood, after endothelial attachment, will traverse the interstitial space and appear in the lymphatic system. Consequently the appearance of tumour cells in the thoracic duct lymph after injection into the blood stream may be correlated to the ability of the cells to stick to the endothelium and to traverse the interstitial space. The thoracic duct was cannulated in female SIV-50 rats.*[51] *Cr-labelled Walker Carcinosarcoma 256 cells were injected via a polyethylene cannula from the femoral artery into the aorta beneath the diaphragm. Lymph specimens were sampled in intervals of 15 min over a period of 8 hr. Each lymph sample was passed through an 8 μ millipore filter. The presence of tumour cells was proved by morphological criteria after Papanicolaou staining and by scintillation counting of the filter. Normal animals show an initial slope of radioactivity beginning 15 min after tumour cell injection. Heparinization of the animals resulted in the loss of the initial tumour cell slope. Various viability tests showed that the heparin concentration used was not cytopathic. Since the clearance of tumour cells from the blood was not different in heparinized and non-heparinized rats, it is assumed that our results reflect a decreased interstitial transmigration of the tumour cells in the heparin group.*

THE INFLUENCE of heparin upon the spreading of experimental tumours has been investigated by several authors; however, many of the reported results are contradictory [1–7]. The present communication deals with an experimental model designed to give quantitative information about the earliest phase of metastasis formation and the action of heparin upon it. Figure 1 schematically demonstrates the experimental arrangement. Walker Carcinosarcoma 256 cells injected into the blood stream of rats will either traverse the capillary bed or, after endothelial attachment, penetrate the vessel wall into the interstitial space. There the cells may (1) remain as dormant cells, (2) die, (3) form a metastasis or (4) traverse the extravascular space and reach the lymphatics. The number of tumour cells in the thoracic duct lymph after injection into the circulation will be an index of cellular penetration of the vessel wall and the extent of transmigration through the interstitial space.

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*Substantial parts of the present paper were submitted by L. Beyerle to the Faculty of Medicine at the University of Ulm as M.D. thesis.

MATERIAL AND METHODS

Four days after intraperitoneal inoculation of approximately 8×10^6 Walker Carcinosarcoma 256 cells into female SIV–50 rats (100–120 g) the tumour ascites was harvested for the experiments. The ascites cells were separated from red cells by several washings in buffered saline (pH 7·4) and differential centrifugations. Finally the tumour cell suspension was adjusted to approximately 10^6 cells/ml. Twenty ml of the suspension were incubated with 100 μ Ci ^{51}Cr ($Na_2\ ^{51}Cr\ O_4$) for 1 hr at 37°C in a water bath with a mechanical shaker. After incubation the cell suspension was subjected to repeated refrigerated centrifugation until the radioactivity of the supernatant had decreased to 2000–4000 Cpm/ml. The suspension was

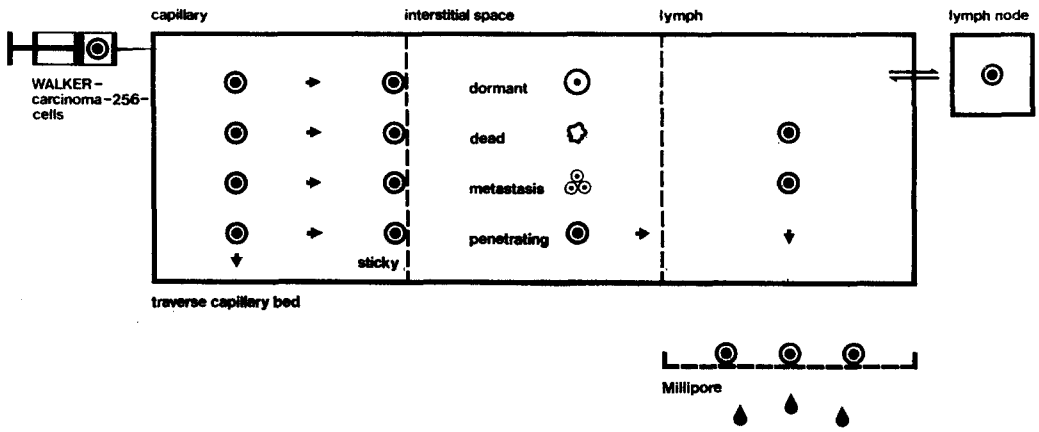

Fig. 1. Fate of tumor cells injected into the blood circulation. Their appearance in the lymphatic system will be preceeded by the transmigration of the vessel wall and the penetration of the interstitial space.

adjusted to 10^6 cells/ml again, containing between 126,000 and 47,500 cpm/ml. The investigations were carried out in female SIV–50 rats weighing 250 to 300 g. After thiobarbital anaesthesia the abdominal part of the thoracic duct was cannulated with a polyethylene cannula which was continously perfused with an EDTA–saline mixture to prevent clotting of the lymph. Subsequently a polyethylene cannula was inserted into the aorta via the right femoral artery and perfused with saline until the actual beginning of the experiment. The end of the cannula was located just below the diaphragm. After these procedures the animals were kept in semirestraining cages.

Three to 4 hr after thoracic duct cannulation, 1 ml of the labelled tumour cell suspension was injected into 2 animals via the aortic cannula. One animal had received heparin intravenously 15 min prior to the tumor cell injection. The same volume of saline was applied to the control animal. Half a USP-Unit of heparin (La Roche, Basle) per g of body weight was sufficient to prolong the animal's whole blood-clotting time 3 to 4 times normal.

Following the intra-aortal injection of the tumor cells the lymph was sampled in different test tubes at intervals of 15 min and kept in an ice bath. One tenth ml of each lymph sample was removed and, after dilution and formalin fixation, passed through an 8 μ millipore filter according to the technique described by Fisher and Fisher [8]. The filter was stained by the method of Papanicolaou [9] and screened for tumor cells under the microscope. Another specimen (0·3 ml) was removed from the lymph sample and kept at 4°C.

Four of these specimens were summarized to a 1 hr sample and 0·5 ml of this lymph were injected subcutaneously into two healthy SIV–50 rats (100 to 120 g). The rest of the lymph sample (between 0·5 and 0·8 ml) was diluted and passed through 5–8 8 μ millipore filters. After rinsing the filters with saline they were assayed together for radioactivity in a scintillation counter.

To ascertain the viability of the injected tumor cells, suspensions containing more than 5% trypan blue stained cells were discarded. Half a ml of the labelled tumour cell suspension (containing 5×10^5 cells) used in the experiments was injected intramusculary into two healthy SIV–50 rats (100 to 120 g). The experiment was neglected if the recipient animal did not develop solid tumors and expire on day 10 after the inoculation, corresponding to the growth pattern of a solid tumour induced by 5×10^5 freshly obtained Walker Carcinosarcoma 256 cells.

In 14 healthy SIV–50 rats (250 to 300 g) the aorta was cannulated. Seven animals were treated with heparin, the rest served as controls receiving the same volume of saline. Thirty min after the intra-aortal injection of 1 ml of labelled tumour cell suspension containing 10^6 tumour cells and 230,000 cpm the animals were bled by aortal puncture.

Five ml of the citrated blood of both groups were diluted with 50 ml buffered saline and passed through 20 8 μ millipore filters. Thereafter the filters were evaluated for radioactivity.

Half a ml of a freshly prepared tumour cell suspension containing 10^6 cells was incubated with 0·5 ml buffered saline containing 1·5 USP–Units of heparin for 1 hr at 37°C. The viability of the cells was tested by trypan blue staining and the intramuscular inoculation of 0·5 ml of the suspension (5×10^5 cells) into two healthy SIV–50 rats (100 to 120 g).

RESULTS

The distribution of the lymph radioactivity on the millipore filters over a period of 8 hr is demonstrated in Fig. 2 and 3. It is obvious that all six control animals show an initial slope of radioactivity beginning 15 min after tumour cell injection. In the corresponding heparin-treated animals this slope is lacking constantly. Approximately 1·5 hr after the cell injection the lymph radioactivity of the control group reaches the level of the heparin group. Since two animals were always treated in exactly the same way, exept that one of them had received heparin prior to the labelled tumour cell injection, the difference of radioactivity on the filters was interpreted as different tumour cell content of the lymph samples in the two groups.

The difference of tumour cell content was also confirmed by morphological criteria. Although a quantitative evaluation of the filters by means of tumour cell morphology was not possible, it was easy to detect tumour cells during the first hour after the tumour cell injection in the lymph samples of the control animals (Fig. 4). In the heparin group and the following period of the control group tumour cells were hardly detectable. No difference in the content of other cellular material in the lymph was found between the two groups. Subcutaneous injection of 0·5 ml lymph of the control animals collected during the first hour after intra-arterial injection resulted in tumour growth in 80 % of the rats, whereas the lymph of heparinized animals induced solid tumours only in 15 % of the recipient animals. These data give further evidence for the accuracy of the results obtained by the isotope technique.

The intra-aortal injection of a constant number of Walker Carcinosarcoma 256 cells resulted in a similar tumour cell count in aortic blood of the two groups 30 min after the injection. Table 1 shows the filter radioactivity

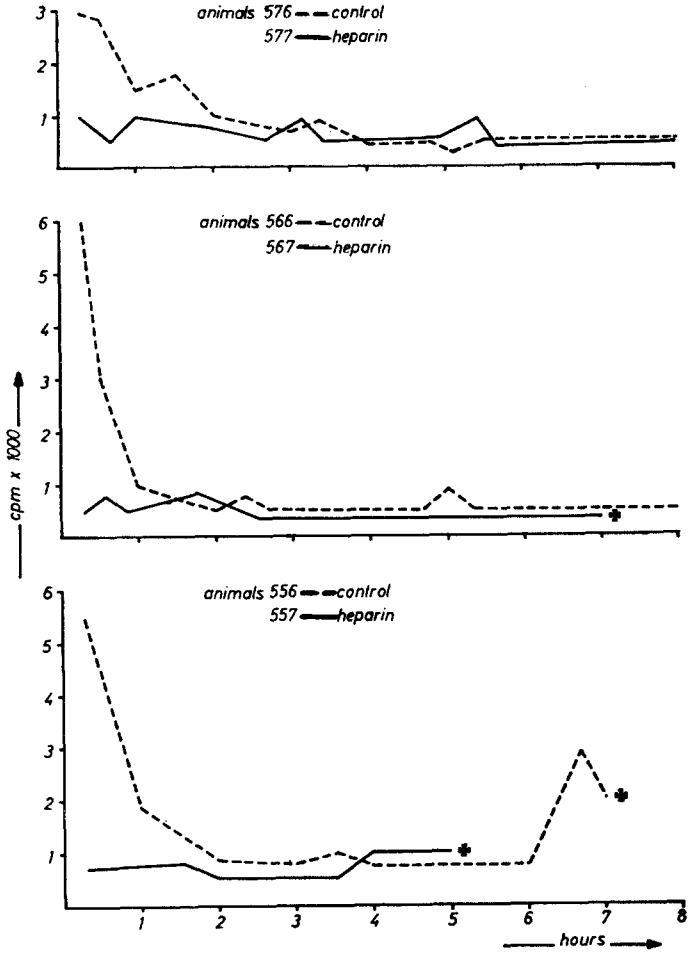

Fig. 2. Distribution of the lymph radioactivity on millipore filters in control and heparinized rats following the intra-aortal injection of ^{51}Cr-labelled Walker Carcinosarcoma 256 cells (injected radioactivity: animals 576/577 : 62,500 cpm, 566/567 : 78,750 cpm, 556/557 : 70,000 cpm).

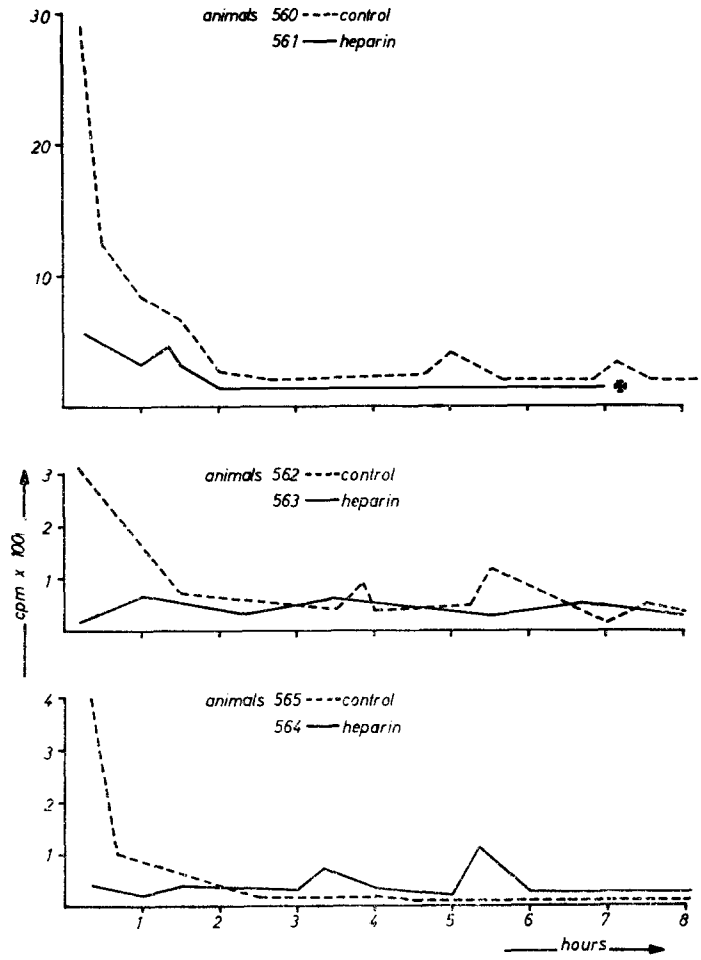

Fig. 3. Distribution of the lymph radioactivity on millipore filters in control and heparinized rats following the intra-aortal injection of ^{51}Cr-labelled Walker Carcinosarcoma 256 cells (injected radioactivity: animals 560/561 : 126,000 cpm, 562/563 : 57,000 cpm, 565/564 : 47,500 cpm).

of equal volumes of blood from the two groups. It is evident that there is no statistical difference between the heparin and the control group.

Control (cpm)	Heparin (cpm)
1910	1580
1220	2180
2140	1440
1710	1980
2180	1760
1780	1690
1410	2050
Mean 1760	1810

Table 1. Blood radioactivity on millipore filters in control and heparinized animals 30 min after the intra-aortal injection of ^{51}Cr-labelled Walker Carcinosarcoma 256 cells (injected radioactivity: 230,000 cpm/animal)

With the methods used, the incubation of 10^6 Walker Carcinosarcoma 256 cells with 1·5 USP–Units of heparin did not show any significant cytopathic effect of this heparin concentration. The amount of trypan blue stained cells remained under 5 % and the intramuscular injection of the heparin incubated tumour cell suspension resulted in tumour growth and the animals' death within 10 days in 100 %.

DISCUSSION

Since in untreated rats mesenteric lymph nodes do not represent an effective barrier for Walker Carcinosarcoma 256 cells [10] the quantitative recording of tumour cells in the thoracic duct may be related to their ability to attach to the endothelium, penetrate the vessel wall and pass the interstitial space. In the experimental model described the tumour cells were injected into the aorta just prior to the branching off of the mesenteric arteries. In comparison to intravenous inoculation of the tumour cells the advantage of this procedure was the avoidance of the lung as a primary site of tumour cell retention which would be less suitable in the experimental model used.

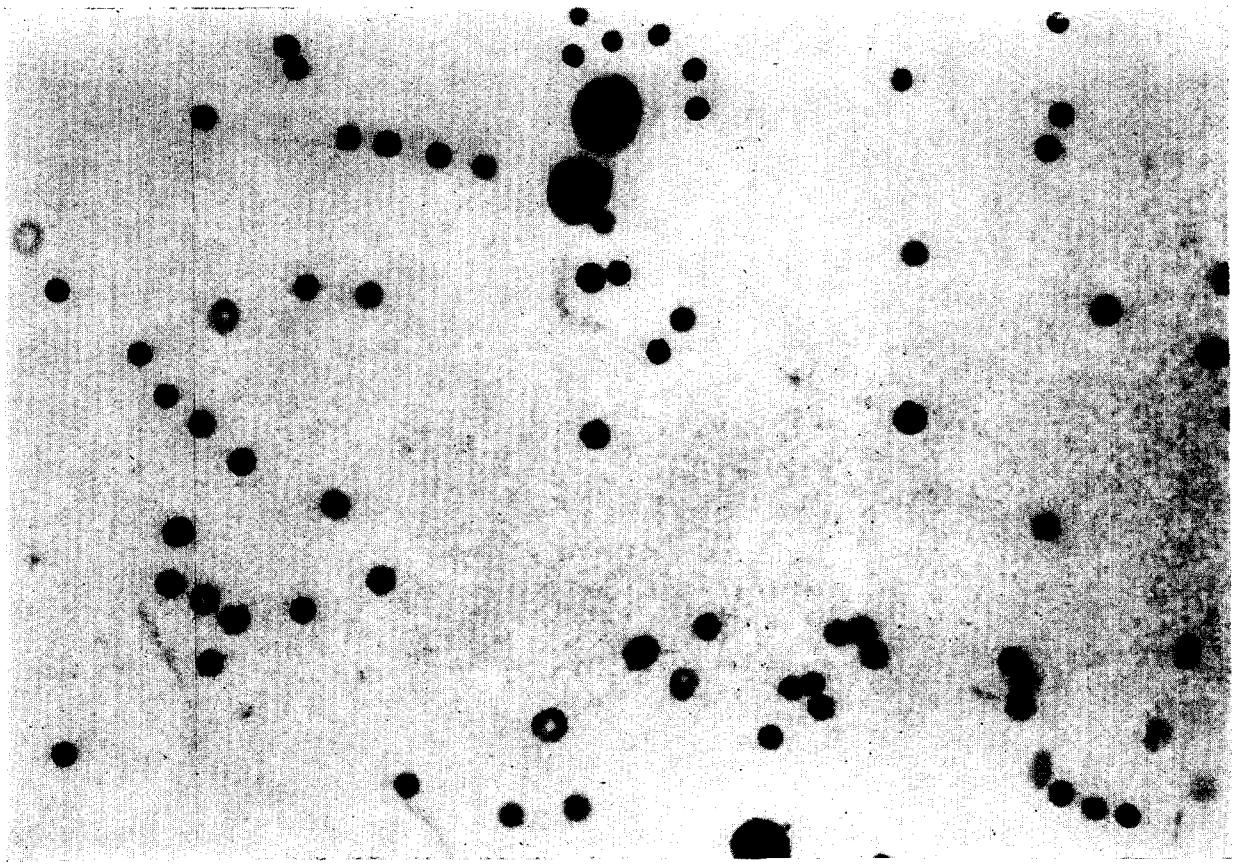

Fig. 4. Lymph sample of a control animal taken 15 mins after intraaortal tumor cell injection and passed through a millipore filter (Papanicolaou staining).

(to face p. 350)

Although cell labelling with ^{51}Cr has been widely used for metastasis studies [8, 11] it was subjected to severe criticism [12]. In the experiments described, the results obtained by the isotope technique are in good accordance with the morphological proof of tumour cells on stained filters and with the results of the bioassay after subcutaneous inoculation of the lymph into healthy recipient animals. This fact allows quantitative statements concerning the tumour cell content of the lymph.

Already 15 min after the injection the maximum of the lymphatic tumour cell content is registered. This is in accordance with the investigations of Griffiths and Salsbury [13] who demonstrated extravascular tumour cells as early as 5 min after intravenous injection of 10^5 Walker Carcinosarcoma 256 cells. Similar results were obtained by Fisher and Fisher [8]. While the initial tumour cell slope of the lymph is completely suppressed by heparinization of the animal, the disappearence rate of the tumour cells from the systemic circulation seems not to be altered. These results may suggest that heparin affects the ability of the cells to traverse the interstitial space and/or lymph nodes with subsequent entering of the lymphatic system. The mechanism of the heparin induced extravascular tumour cell retention remains obscure.

A cytopathic effect of the heparin dose used in the experiments is unlike since the *in vitro* pre-incubation with 1·5 USP–Units of heparin —a higher concentration than is achieved in the *in vivo* experiments—did not influence the uptake of trypan blue by the cells and the growth pattern of the transplanted cells was not altered.

Many investigators have established the antimetastatic effect of heparin in experimental tumours [1–3, 7]. Microcinematographic studies indicated that tumour cells after endothelial attachment were surrounded by a fibrin and platelet clot [14]. Under protection of this thrombus the tumour cells penetrate the vessel wall. Heparin is supposed to interfere with this fibrin formation and thus may inhibit the development of a tumour cell thrombus.

Fisher and Fisher found an increased tendency to hepatic retention of intraportally injected Walker Carcinosarcoma 256 cells [4]. Twenty four hours after the injection, 23·1 % of the cells were found in the liver of control animals, compared to 28·9 % in the liver of heparinized animals. Recently, Hagmar and Norrby reported that heparin induced changes in haematogenous pulmonary metastases in a syngenic tumour-host system [6]. Pretreatment of the animals with heparin did not affect pulmonary metastases significantly, but when given in the cell suspensions the metastasis volume became consistently smaller and their number had a tendency to increase. The action of heparin was interpreted by the authors as being due to changes of the tumour cell surface.

Heparin is a polymer with a high negative electric charge. Ambrose [15] pointed out that polyanions may alter membrane functions and cellular volumes. Suemasu and Ishikawa confirmed the increased electrophoretic mobility of heparin treated tumour cells [7]. Our own investigations have demonstrated that heparin increases the adhesiveness of various ascites tumour cells to prepared glass surfaces [16]. Up to now there is strong evidence that heparin may affect tumour cell lodgement by mechanisms other than inhibited blood coagulation.

Most likely heparin alters the surface properties of the cancer cell by its polyanion character although other modes of action, i.e. lipoprotein lipase activation [17] or complement binding [18] cannot be ruled out. Since heparin is a potent inhibitor of plasma kallikrein and its kinin-forming activity [19], alterations of the vascular response to tumour cell lodgement may play an additional role. In this context, a possible influence of heparin on lymphaticovenous communications may alternatively be discussed.

In conclusion, the heparin-induced retention of tumour cells in the extravascular space, as demonstrated by their lack of appearence in the thoracic duct lymph, is difficult to explain exclusively by changes of the coagulation mechanism of the host animal. However, our results further support the statement that heparin may have an effect on tumour cell surfaces even *in vivo*.

Acknowledgements—This work was supported by grants from the Landesverband Württemberg zur Erforschung und Bekämpfung des Krebses.

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A Randomized Study of Irradiation and Vinblastine in Stages I and II of Hodgkin's Disease

Preliminary Results

RADIOTHERAPY–CHEMOTHERAPY COOPERATIVE GROUP* of E.O.R.T.C.

THE MAIN objective of the clinical trial, whose preliminary results are reported here, was to answer the question: would addition of long term chemotherapy improve results of radical radiotherapy in stages I and II of Hodgkin's disease.

This trial was carried out by the E.O.R.T.C. Radiotherapy—Chemotherapy Cooperative Group. Fourteen hospital centres (6 French, 5 Dutch, and 3 Belgian) participated.

Accepted 18 November 1971.

*The chairmen of this cooperative group have been: M. Tubiana (1964–1967), K. Breur (1967–1969), B. van der Werf-Messing (1969–1971). A. Laugier (General Secretary); M. Hayat (Statistician).

Cooperative Centres have been in France: C.A.C. Bordeaux (C. Lagarde), C.A.C. Caen (S. Abbatucci), Lyon (J. Papillon, L. Revol), Paris: Hôtel-Dieu (J. Bousser), C.A.C. Rouen (H. Piguet), Villejuif (G. Mathé, M. Tubiana, J. L. Amiel). In Belgium: Brussels Institut Jules Bordet (J. Henry), Liège (R. Lemaire), Louvain (A. Wambersie). In Holland: Amsterdam (K. Breur, M. Burgers), Leiden (P. Thomas), Nijmegen (C. Haanen), Rotterdam (B. van der Werf-Messing, W. F. Stenfert-Kroese).

The members of the histology review committee: R. Gérard-Marchant, J. van Unnik; and of lymphography review committee: R. W. Kropholler, J. D. Picard, P. Markovits, J. L. Chassard.

Address for reprints: M. Hayat, M.D., Unité de Recherches Statistiques, Institut Gustave-Roussy, 94—Villejuif, France.

PATIENTS AND METHODS [1]

Patient selection

Patients were included in the trial if the following conditions were met: (*a*) the diagnosis was confirmed by biopsy and cytologic examination; (*b*) the patient had not received previous treatment for this disease; (*c*) the patient was judged to be in stage I or stage II after a careful clinical and radiological examination, which included: a successful ilio-lumbar lymphangiogram; X-ray of the mediastinum and tomography; liver function tests and, if indicated, liver biopsy.

Radiotherapy

Radiotherapy was carried out by telecobaltherapy according to a technique based on that of Kaplan [2]. The whole target volume is irradiated in a single field, which is defined by lead screens so that areas of demonstrable disease, as well as adjacent and intervening areas containing lymph nodes, are included. However, if the mediastinum is involved, the lumbar nodes are not irradiated, and conversely, if the lymphangiogram indicates involvement of the lumbar nodes, the mediastinum is not irradiated. The diaphgram is always the upper or lower limit of the irradiated volume.

A dose of 3500–4000 rads is delivered in 4 weeks, 1000 rads/week in 3 sessions of 330 rads each. Anterior and posterior fields are used alternately. If the tumour mass is large, a

small supplementary field is superimposed to raise the tumour dose to 4500 rads.

Four to 6 weeks after the end of the irradiation, those patients who are in complete remission are randomly assigned to 2 groups: (*a*) patients who receive no further treatment; (*b*) patients who receive a weekly injection of vinblastine.

Chemotherapy

Vinblastine is injected each week in a dose of 10 mg if white blood cell count is above 5000/mm^3 and of 5 mg if white blood count is between 3000 and 5000/mm^3. If below 3000/mm^3, the injection is postponed for one week. This treatment with vinblastine is continued for 2 years.

Patients of the two groups are examined regularly every 2 months for 6 months, every 3 months for another 6 months and then every 6 months till relapse. The criterion chosen for comparing the two groups is the duration of the first remission. As soon as relapse occurs, each Centre is free to decide the further treatment to be used.

Number of patients to include in this trial was calculated depending on following assumptions: with radiotherapy alone it was estimated that 60 % of patients would be still in apparent complete remission after 3 years. It was contemplated that, with chemotherapy, this percentage could be increased to 80 %. With α and β, the probabilities of a false positive and a false negative, being 0·05 and 0·05 respectively, the necessary number of patients is 110 in each group.

The clinical trial began in July, 1964. The preliminary results reported here are up to May, 1971. Fifty six per cent of the patients with Hodgkin's disease treated in the Cooperative Centres during this period, i.e. 387, were within the criteria for inclusion and received radiotherapy treatment as defined in the protocol. Among these 387 patients, 23 % were not randomized because they were not in complete remission 6 weeks after the end of radiotherapy.

RESULTS

This is a progress report and the results cannot be considered as final yet. Two hundred and ninety-five patients were randomized in the Cooperative Centres. Breakdown of patients for each Centre and according to the treatment is shown in Table 1. Twenty two patients were lost to follow-up. They came mainly from three Centres and are equally distributed in the two groups; it is estimated their loss cannot influence the results. We later excluded 5 patients which the members of the histology review committee did not consider as having Hodgkin's disease.

Table 1. *Distribution of patients included in the trial according to centres and treatment*

Centres \ Groups	Radio-therapy alone	Radio-therapy with chemo-therapy	Number of patients
Amsterdam	16	14	30
Nijmegen	3	3	6
Rotterdam	44	42	86
Leiden	12	14	26
The Hague	1	0	1
Brussels (Institut J. Bordet)	5	6	11
Louvain	1	0	1
Liège	4	3	7
Lyon	5	5	10
Bordeaux	3	1	4
Caen	7	9	16
Paris (Hôtel-Dieu)	11	8	19
Villejuif (Institut Gustave-Roussy)	35	35	70
Rouen	1	2	3
Number of patients	148	142	290

The two groups were compared on the basis of various criteria. Figures 1, 2 and 3 show there were no significant difference in their composition with respect to the age distribution of the patients, their sex, their clinical stage or tumour histology. As regards histological material, slides for only 205 patients were reviewed and classified by at least one of the two pathologists of the group.

Analysis of results was carried out from all the follow-up forms completed every 6 months. Comparisons of the first complete remission durations within the two groups were calculated by the actuarial method (Fig. 4). There is a significant difference in favour of the group which received chemotherapy. In the latter group, it is estimated that the 1-year recurrence rate is 8 %, compared with 24·5 % for the group with radiation-therapy alone. This difference, nearly constant until the 42nd month, is no longer significant at the 48th month. However, it should be noted that only 82 patients have been followed up for 4 years. It should also be kept in mind that chemotherapy was discontinued at the end of a 2-year period.

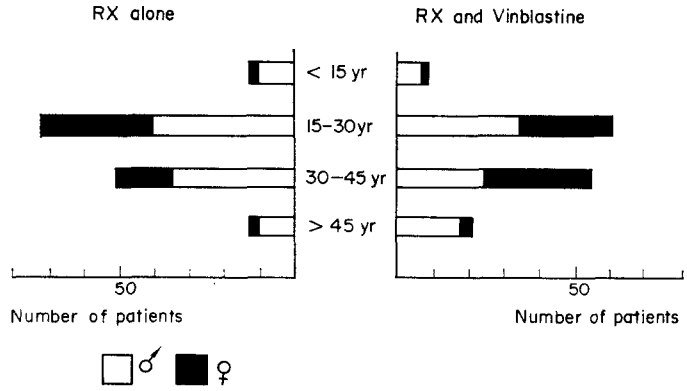

Fig. 1. Distribution of patients according to sex and age.

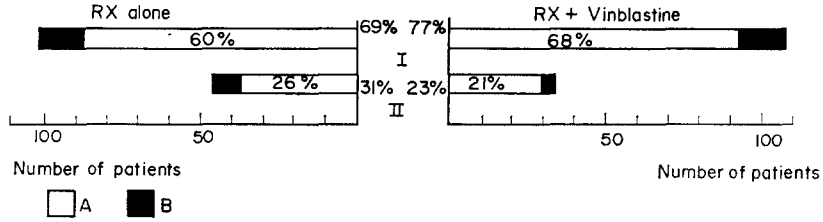

Fig. 2. Distribution of patients according to stage and systemic symptoms.

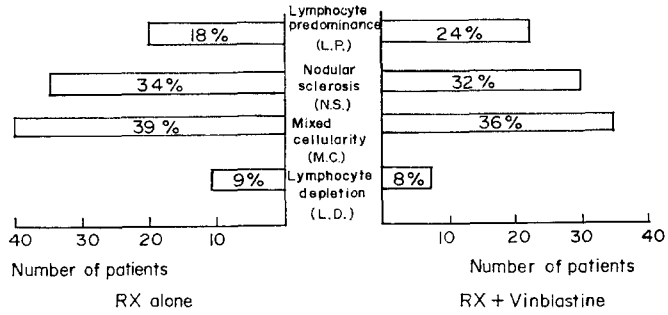

Fig. 3. Distribution of patients according to histological type.

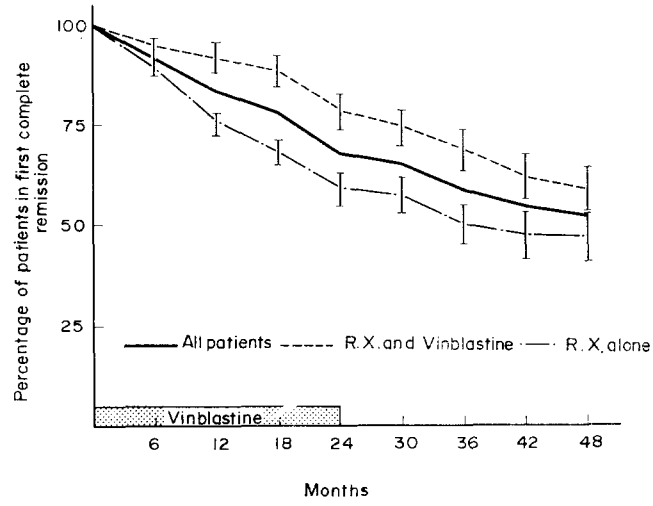

Fig. 4. Percentage of patients in first complete remission according to treatment (actuarial curve).

The actuarial survival curves are shown in Fig. 5 and indicate no significant difference up to 4 years. However, the trend is in favour of the group treated with chemotherapy. It is to be noted that survival was not the principal end point in this clinical trial and that when a relapse occurred the patients were treated differently in each Centre.

Analysis of results according to Centres

We attempted to verify if the frequency of relapses differs from Centre to Centre. Occurrence of relapses is significantly less within Dutch Centres than within others (Fig. 6). This difference is significant at 12 and 24 months of follow-up but is no longer significant after 36 months. Comparison of the two therapeutic groups for the patients treated in the Dutch Centres does not reveal any difference in favour of chemotherapy, whereas the difference remains significant for the patients treated in the other Centres. This raises the question why there were fewer relapses in patients attending the Dutch hospitals, and why there was no difference in the response to the two forms of treatment in the Dutch patients. This could have been due to variations in the precise interpretation of the treatment protocol in the different Centres or, the Dutch patients, before any treatment, have a better prognosis.

It is to be further noted that by reducing the number of patients, the comparison of the two treatments becomes more difficult as small differences may not be noticeable any longer.

Comparison of treatments according to Centres

The average duration between the first symptom and beginning of radiation therapy did not differ according to Centres: in Netherlands 5·7 months; in other Centres: 5·1 months. The duration between end of radiation therapy and randomization did not differ according to Centres: in Netherlands, 6·9 weeks; in other Centres: 6·8 weeks.

Three patterns of relapses have been distinguished: (*a*) the relapses in an irradiated area; (*b*) the relapses in a non-irradiated area; (*c*) the relapses occurring with systemic symptoms or visceral localization. Table 2 shows the distribution of these relapses at the end of 2 years

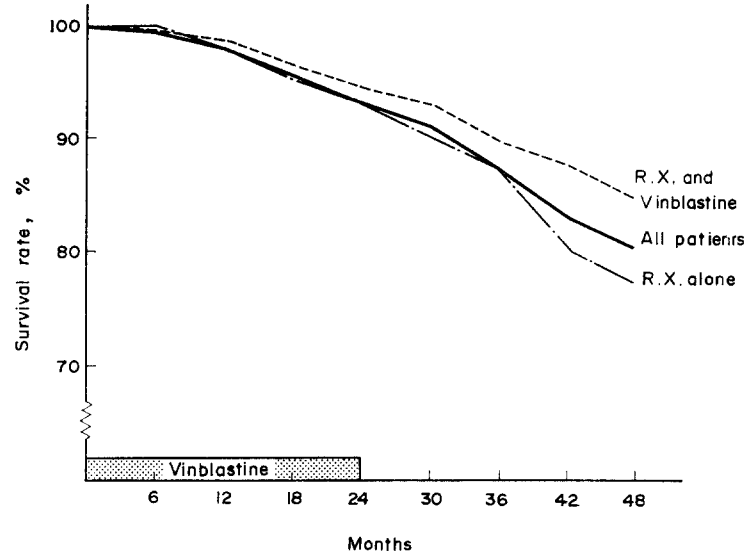

Fig. 5. *Survival rates according to treatment (actuarial curve).*

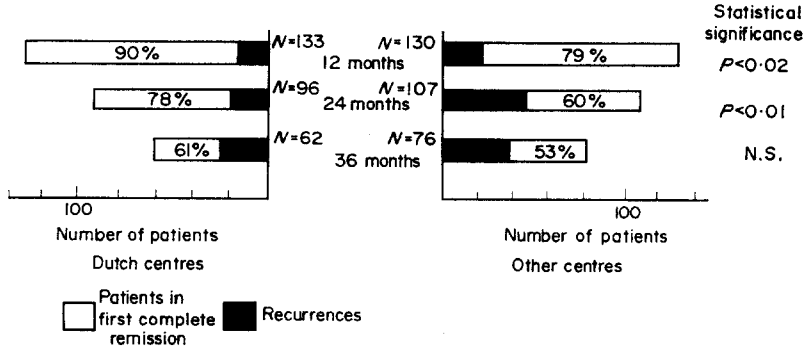

Fig. 6. *Distribution of recurrences according to therapeutic centres.*

and also according to the treatment. The difference in favour of the group with chemotherapy is observed only for the relapses in non-irradiated areas. Comparison of relapses according to Centres shows there is no modality of relapse more frequent in certain centres. In particular the incidence of relapses in an irradiated area is similar in all Centres (5 % of the patients in Dutch Centres, 4 % in other Centres). That leads us to suppose that efficiency of radiotherapy has been roughly the same in all Centres.

Table 2. *Modalities of recurrences according to treatment of patients with a* 2*-year follow-up*

	Patients with radiotherapy alone $N=94$ (%)	Patients with radiotherapy and vinblastine $N=87$ (%)	Statistical significance
Recurrences in an irradiated area* (involved or contiguous)	9·5	10·5	N.S.
Recurrences in a non-irradiated area (same side or other side of the diaphragm)	20	3·5	$p < 0{\cdot}001$
Other recurrences (visceral or systemic symptom)	15	12·5	N.S.

*Area clinically involved or adjacent to a clinically involved area.

Study of prognostic factors

Treatments being probably comparable in Dutch Centres and other centres, we wondered whether the difference found between the results was not due to prognosis factors.

(1) *Sex.* After 36 months follow-up, the relapse percentage is significantly lower for women than for men (28 %, 46 % respectively); but males and females are equally distributed in each group, in each of the Centres.

(2) *Clinical stage.* The breakdown of stage I and II is similar in Dutch centres and in other Centres, but there are fewer patients with systemic symptoms in Dutch Centres: 7 % vs. 21 % in the other centres. However no difference was observed in the duration of the first remission according to stage I or II or according presence or absence of systemic symptoms.

(3) *Age.* The number of patients under 30 years of age is significantly more important for Dutch Centres (Table 3). Sixty per cent of the patients are under 30 years of age in Netherlands against 45 % in the other centres. Figure 7 shows that relapse is less frequent in patients under 30 years of age. Analysing the effect of chemotherapy according to age, it is seen that chemotherapy does not have any effect on patients less than 30 years of age (Fig. 8). It is only effective in patients over 30 years of age (Fig. 9).

Table 3. *Comparison of therapeutic Centres by age*

Age (years)	Dutch Centres n	Dutch Centres %	Other Centres n	Other Centres %
< 30	89	60	61	45
> 30	58	40	76	55

Statistical significance $p < 0{\cdot}01$.

Table 4. *Relation between age and histology*

	Lymphoid predominance n	Lymphoid predominance %	Nodular sclerosis n	Nodular sclerosis %	Mixed cellularity n	Mixed cellularity %	Lymphoid depletion n	Lymphoid depletion %
Patients less than 30 years old	24	22	44	39	39	35·5	4	3·5
Patients more than 30 years old	20	20·5	23	25	37	39	14	15·5

Statistical significance $p < 0{\cdot}025$.

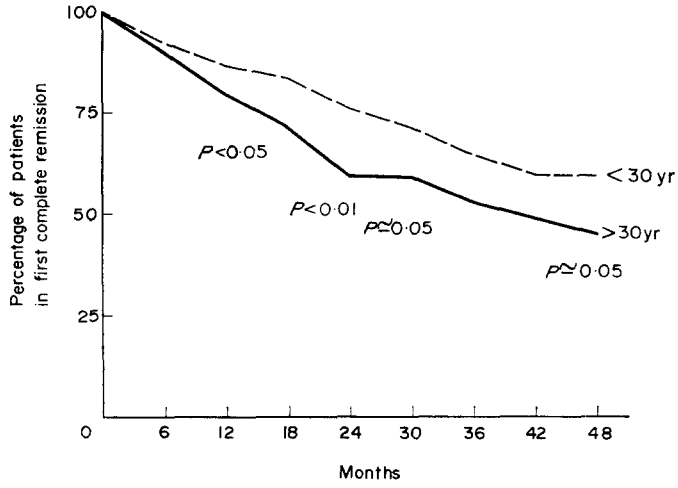

Fig. 7. Percentage of patients in first complete remission according to age (actuarial curve).

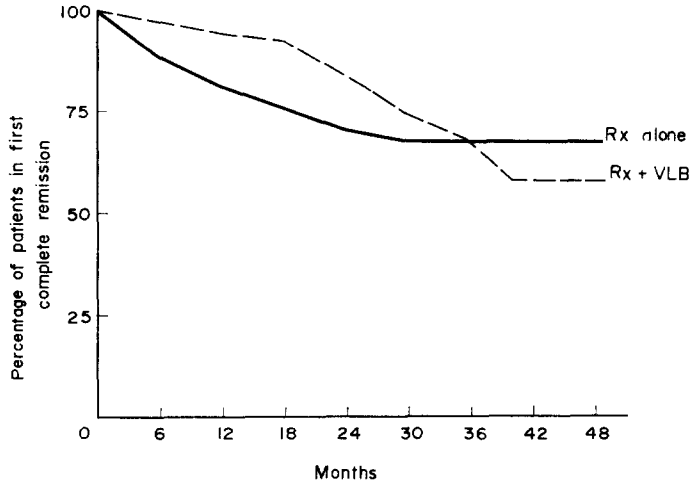

Fig. 8. Percentage of patients under 30 years of age in first complete remission (actuarial curve).

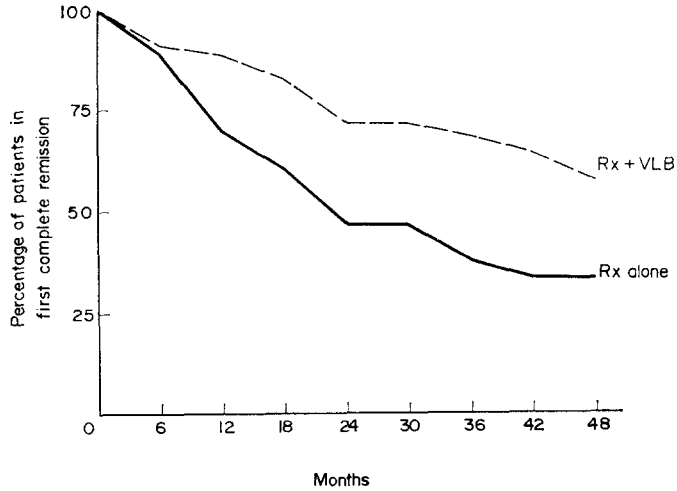

Fig. 9. Percentage of patients over 30 years of age in first complete remission (actuarial curve).

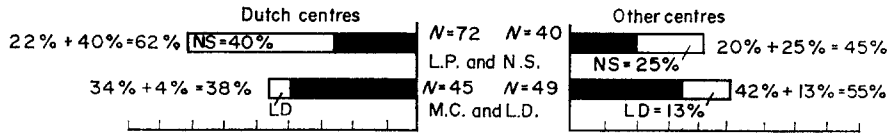

Fig. 10. *Distribution of patients according to the histological type and Centres.*

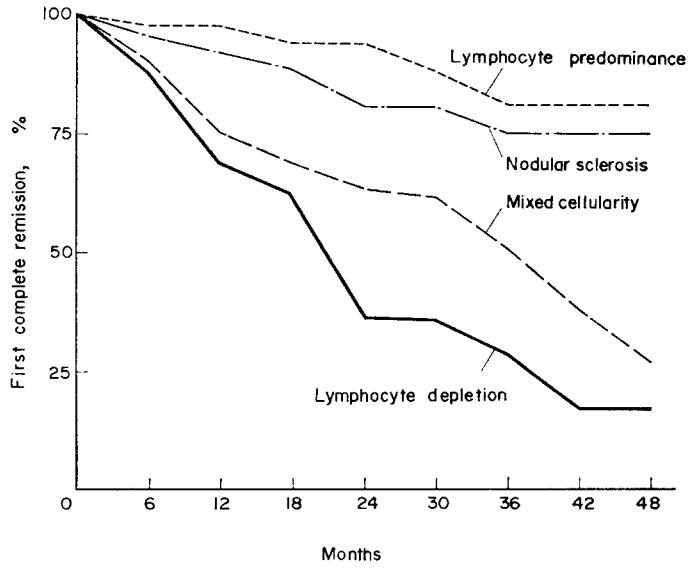

Fig. 11. *Percentage of patients in first complete remission according to histological type (actuarial curve).*

(4) *Histology.* The proportion of patients in each of the 4 histological types varies significantly according to the different Centres (Fig. 10). There are more cases with lymphocytic predominance and nodular sclerosis in Netherlands: 62% compared to 45% in the other centres. Frequency of relapses differs according to histology (Fig. 11). Relapses were more frequent if the histological type was mixed cellularity and lymphocytic depletion. For the histological types "lymphocytic predominance" and "nodular sclerosis", chemotherapy did not seem to have any effect (Fig. 12 and 13), but there is a clear difference in favour of chemotherapy for "mixed cellularity" (Fig. 14). However, in this histological type, from the 30th month onwards, that is 6 months after discontinuity of chemotherapy, there is no longer any difference in the incidence of relapses between both therapeutical groups. However 48 months after the beginning of the treatment the survival rate remained higher in the group treated by long term chemotherapy but the difference is not significant. The number of patients with lymphocytic depletion is too small for a difference to be detected.

DISCUSSION

The discrepancy between the Dutch Centres and the other Centres may have an explanation. Before any treatment, the patients included in the study in Holland were not the same as in the other Centres. Amongst them, there were more patients under 30 years of age, and more forms of lymphocytic predominance and nodular sclerosis types of Hodgkin's disease. Both factors, as confirmed in our study, have a most favourable prognosis. The addition of vinblastine as a chemotherapy, for those forms with a favourable prognosis, does not improve the results of radiation therapy alone.

The reason for the discrepancies of age and histological type between Dutch patients and the others is uncertain. A possible explanation might be differences in the referral of the patients to the treatment Centres, or, more likely, different epidemiological features related to nationality [3].

The analysis has shown a relationship between age and histological type (Table 4): there are more cases with mixed cellularity and lymphocytic depletion amongst patients over 30

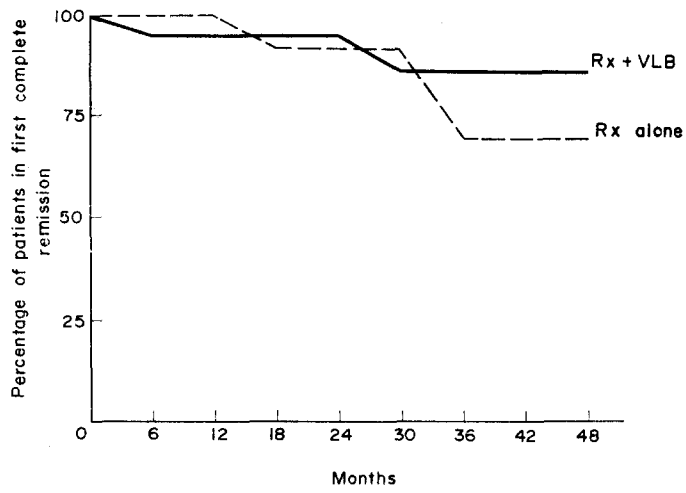

Fig. 12. *Percentage of patients with lymphocyte predominance in first complete remission according to treatment (actuarial curve).*

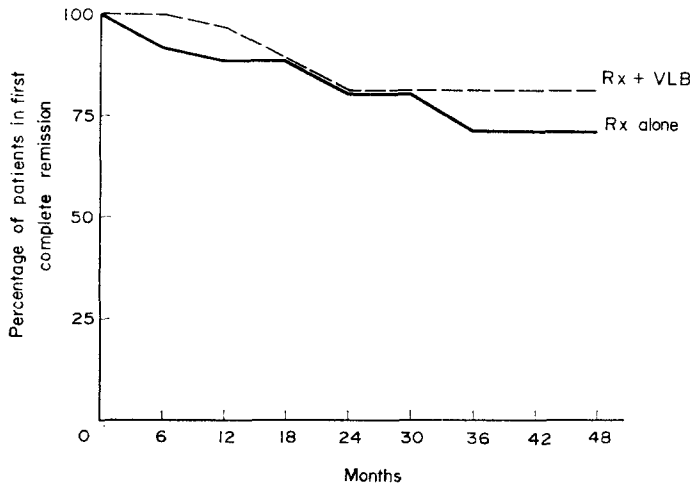

Fig. 13. *Percentage of patients with nodular sclerosis in first complete remission according to treatment (actuarial curve).*

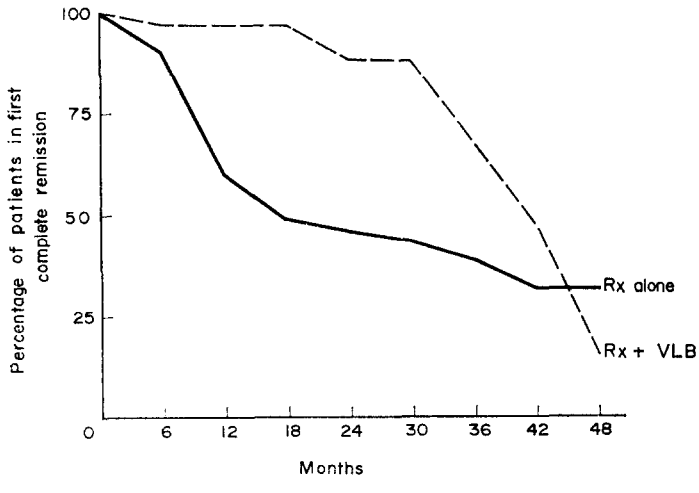

Fig. 14. *Percentage of patients with mixed cellularity in first complete remission according to treatment (actuarial curve).*

years of age (Fig. 14). Histology has a more significant influence than age. We have examined the influence of chemotherapy on the duration of the first remission and survival, according to the histological types, in patients less than 30 years of age and more than 30 years of age. Whatever the age, chemotherapy is beneficial in patients with tumours of mixed cellularity type. This difference holds true until the 30th month after beginning therapy.

This selective effect of chemotherapy was observed when the recurrence was evaluated according to the treatment and histological types (Tables 5 and 6).* The rate of recurrence is similar in both therapeutic groups, for lymphocytic predominance and nodular sclerosis histologic forms. By contrast, for the poor-risk histologic forms (mixed cellularity and lymphocytic depletion), the recurrence rates in an irradiated area and/or non-irradiated area are more frequent amongst patients treated with radiation therapy alone (20 relapses), as compared with patients treated with combined therapy (3 relapses).

Table 5. Modalities of recurrences according to treatment for all patients with histological type of predominance lymphoid and nodular sclerosis

	Patients with radiotherapy alone	Patients with radiotherapy and vinblastine
Recurrences in irradiated area (involved or contiguous)	2	2
Recurrences in a non-irradiated area	4	5
Other recurrences (visceral or systemic symptom)	6	7

Table 6. Modalites of recurrences according to treatment for all patients with histological type of mixed cellularity and lymphoid depletion

	Patients with radiotherapy alone	Patients with radiotherapy and vinblastine
Recurrences in irradiated area (involved or contiguous)	7	1
Recurrences in a non-irradiated area	13	2
Other recurrences (visceral or systemic sympton)	10	9

*Relapses observed in the group of 85 patients whose slides were not reviewed do not appear in these tables.

CONCLUSION

These findings show that at a similar stage, not only the prognosis but the choice of the best type of therapy depends on the patients age and the histological type of the disease. In Hodgkin's disease clinical stages I and II, radiation therapy alone suffices for the lymphocytic predominance and nodular sclerosis histological groups. The addition of a chemotherapy by means of vinblastine, a particularly well-tolerated drug, improves the results for the mixed cellularity and lymphocytic depletion forms.

The preliminary results of this study raise at least two problems.

Do the results obtained with this type of chemotherapy hold true with other chemotherapeutic regimes? For instance, a more intensive chemotherapy of the same type as used by Moxley *et al.* [5] in combining radiation and chemotherapy for the stages I and II. Only a new controlled trial can answer this question.

Since 1964, the medical investigation and treatment procedures in Hodgkin's disease have changed and been developed; the practice of abdominal laparotomy with liver and abdominal nodes biopsies have modified the conditions of initial assessment, and splenectomy constitutes both a diagnostic and therapeutical process. Moreover, experience has shown that irradiating all of the lymphatic areas was possible and fairly well-tolerated, and some therapists are using this type of treatment, either for some categories of stages I and II, or even for all categories of patients. Finally, advances in therapy have been considerable, since the trial was initiated. The conclusions of this study will have to be re-examined, taking into account all of these factors, and the value of chemotherapy in Hodgkin's disease cannot be determined definitively from this clinical trial.

The trial has demonstrated the importance of histological type on the response to therapy in Hodgkin's disease. It will now be necessary to organise further trials to examine the situation once more in the light of recent advances in oncology and chemotherapy.

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Letter to the Editor

Isolation of a C-type Virus from Mouse Myeloma Cells

ABRAHAM KARPAS and JOHN CAWLEY

Department of Medicine, University of Cambridge, Cambridge, England

PLASMA cell tumours producing immunoglobulin in the inbred BALB/c strain of mice can be induced experimentally [1]. Typical myeloma has been produced following the administration of, for example, intraperitoneal (i.p.) implantation of solid materials, such as plastic diffusion chambers, plastic shavings, and circular discs of polymethylmethacrylate [2, 3], and by i.p. injections of white mineral oils [1, 4].

Several papers described the presence of A-type particles in the cytoplasm of the pathological cells [5, 6]. These particles were found recently in tissues of normal BALB/c mice [7]. The virus-like A-type particles were reported to be located singly or in clusters within cisternae of the endoplasmic reticulum [6, 7]. Recently it has been reported that immunoglobulin-synthesizing mouse myeloma cells produce an RNA-containing virus which has both an RNA base composition similar to that of other murine leukaemia viral RNA and the group specific antigen of the murine leukaemia-sarcoma group [8]. Ultrastructural studies of the myeloma cells have revealed extra-cellular C-type particles in addition to the intracisternal A-type particles. However, attempts to demonstrate biological activity of this C-type virus both *in vitro* with a variety of tissue culture lines and *in vivo* with mice and rats, gave consistently negative results [8].

The successful *in vitro* isolation of a biologically active C-type virus in normal mouse fibroblasts of the 3T3 cell line is described in the following report.

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For the isolation of the C-type virus we used the P–3 plasma cells which are a cloned line (originated by Drs. K. Horibata and M. Cohn) derived from the BALB/c strain of mice and known to produce IgG and to contain viral particles [8]. The P–3 cells were cultured in Dulbecco's modified Eagle's medium (DMM) supplemented with 10% horse serum. Some of the P–3 cells were fixed in 1·5% glutaraldehyde for electron microscopy. Other P–3 cells were co-cultivated with 3T3 cells (mouse embryo fibroblast line) and the tissue culture fluid from the mixed culture was passed through a 0·45 μ millipore filter, and this filtrate was used to infect 3T3 cultures. Both the infected 3T3 cells and non-infected control cells were trypsinized and subcultured every 10 days. Following the fifth passage infected and control 3T3 cells were seeded in 50 mm plastic plates and incubated in air with 5% CO_2 at 37°C. After 24 hr 25 μg/ml DEAE–D was added in order to render the cells more susceptible to viral infection [9]. Following the DEAE–D treatment a suspension of the Harvey strain of murine sarcoma virus (MSV–H) was inoculated into infected 3T3 and control cells. After incubation of 1 hr DMM containing 5% calf serum was added to the plates and the cultures were placed in the CO_2 incubator. The control 3T3 cells infected by MSV–H developed within 4 days numerous foci of transformed cells characteristic of the MSV. In contrast, none of the 3T3 cultures which had been infected earlier with P–3—3T3 co-cultivation filtrate developed any foci of cell transformation. This observation was repeated three times. The resistance of the infected 3T3 cells to super-

infection by MSV is in line with previous observations which indicated that mouse embryo fibroblasts previously infected with any of the murine leukaemia viruses become resistant to super-infection and transformation by the MSV [10]. It suggested therefore that a leukaemia type virus replicated in the 3T3 cells.

In order to confirm that a replicating C-type virus is responsible for the resistance of 3T3 cells to infection by MSV, the myeloma virus infected cells and their non-infected control 3T3 cells were processed for electron microscopic examination. Examination of the ultrastructure of the infected 3T3 cells, showed that the cells produce a very large number of mature C-type particles, 100 mμ in diam. (Fig. 1) thus confirming the biological observation. The virus particles were seen only outside the cell membrane. In contrast, the control 3T3 cells contained no virus particles. It was interesting to note that A-type particles were not detected in any of the 3T3 cells which produced C-type virus, whereas the original myeloma cells (P–3) contained numerous intracisternal A-particles 70 mμ in diam. similar to the particles described earlier, and C-type extracellular particles (Fig. 2) which were much less numerous than in the infected 3T3 cells. The C-type virus is probably the one which infected and was readily replicating in the 3T3 cells. The A-type particles evidently do not "infect" or replicate in the 3T3 cells. This is in agreement with earlier work which failed to demonstrate any biological activity associatep with the A-particle [6].

The isolation of the C-type virus in 3T3 cells and its massive replication in these cells will allow the harvesting of high concentrations of virus; such preparations may allow experiments to induce myelomatosis in mice.

The repeated observations that a wide range of carcinogens can induce the same type of malignancy in mice may suggest that these substances activate the "switched off" vertically transmitted C-type virus. Experimental evidence in support of the hypothesis of co-carcinogenesis of chemical or physical agents together with C-type viruses has been accumulated in recent years [11]. Therefore a similar mechanism of co-carcinogenesis may also operate in the induction of myeloma in mice.

Acknowledgments—We thank Dr. C. Milstein for the supply of P–3 cells, and Professor F. G. J. Hayhoe for his helpful comments. The work was supported by the Leukaemia Research Fund (U.K.). John Cawley gratefully acknowledges the support of the Medical Research Council.

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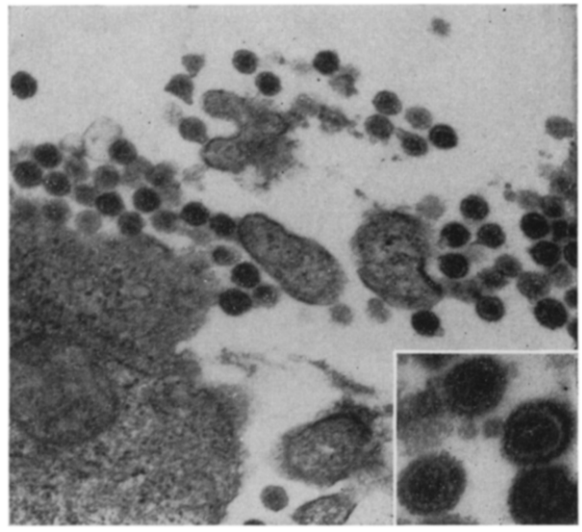

Fig. 1. *Extracellular C-type virus particles in* 3T3 *culture* (× 48,000). *Inset* ×90,000.

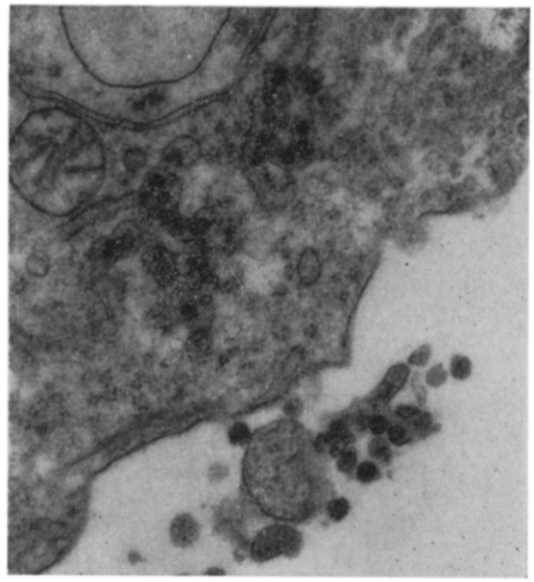

Fig. 2. *P-*3 *cells containing intracisternal A-type particles in the cytoplasm and C-type particles outside the cell membrane* (× 37,000).

(to face page 364)

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Announcement

An international IUPAC-sponsored symposium on *Control of Mycotoxins* is planned to be organized by IUPAC Food Contaminants Commission (FCC) in Göteborg (Sweden) during August (21 and 22), 1972. FCC includes a group of experts working on methods for the analysis of mycotoxins under the guidance of Dr. I. Purchase, Pretoria, South Africa.

The emphasis of the symposium will be on mycotoxins other than aflatoxins which are coming to light as potential hazards to man and animals as Sterigmatocystin, Citrinin, Ochratoxin etc. There are, however, also aspects of the aflatoxins such as the M Aflatoxins, Aflatoxin P and alteration products formed from aflatoxins which are pertinent subjects of this meeting.

Members of FCC are expected to present information from their work and from their parts of the world. In addition rounded representation of current mycotoxin problems by other authors is solicited and international well-known experts are being invited to attend.

Information can be obtained through Dr. R. Marcuse, Chairman of FCC, c/o SIK, Fack, S-400 21 Göteborg 16, Sweden.

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Protocol for an Evaluative Study of the Protective Effect of Isolation Systems and Decontamination in Patients With High Susceptibility to Infection

E.O.R.T.C.* GNOTOBIOTIC PROJECT GROUP

Abstract—*In the fall of* 1968 *Dr. T. M. Fliedner (University of Ulm, Germany) took the initiative to invite a number of clinicians and scientists working in the field of gnotobiology to discuss the necessity of cooperation. Since it would require many years for each individual clinical unit to evaluate the benefit of the isolation as well as antibiotic decontamination of the digestive tract of patients with a strong immune suppression, it was generally recognized that only co-operation could lead to a conclusive study. A close contact between clinicians and scientists experienced in gnotobiology would be of help since a number of questions arising in the clinical application, could be investigated first in experimental animals.*

This original nucleus extended rapidly. In the course of 2 years the protocol, published here, was prepared in a series of eight meetings. During this period the group was recognized as an official project group of the European Organization for Research on Treatment of Cancer (EORTC). Since the final version of the protocol in January 1971, 22 *patients have been randomized. Not all units are at this moment in the position to isolate patients, but are expected to do so within the first half of* 1972.

1. INTRODUCTION

THE USE of an isolation system in the treatment of human patients is based on the fact that in a number of diseases and for certain therapeutic regimens there is a reduction in the patient's resistance to microbial infection. The causative agents of such infections may originate from the patient's own microbial flora or may come from the environment. Consequently, control measures are directed both to eliminate pathogens and potential pathogens from the patient's flora and to protect him from the invasion of dangerous microorganisms in his environment. Antibiotic therapy can usually be employed successfully in achieving the former but may not be adequate for the latter; i.e. antibiotic treatment which controls or eliminates the pathogens and potential pathogens from the patient's flora may be ineffective against pathogens arising from the environment. In a hospital setting particularly, there is a high risk of contamination by antibiotic-resistant organisms as well as viruses, yeasts, and fungi. In addition, in antibiotic-treated patients there is the phenomenon of an increased susceptibility to certain organisms because of a decrease in competitive ecological control (colonization resistance). Thus isolation of the patients seems the procedure of choice in a number of cases, along with decontamination when a bacteriological survey indicates the presence of pathogens and potential pathogens in the patient's flora.

Accepted 25 October 1971.

*European Organization for Research on Treatment of Cancer.

The following protocol, therefore, was developed to define *minimum requirements* in the surveillance of human patient isolation systems. In the opinion of the members of the E.O.R.T.C. Gnotobiotic Project Group, acceptance of these is basic to the successful operation of an isolation system. Unless these requirements are observed, there is little hope that meaningful data can be obtained about the effectiveness of the barrier and the consequences of antibiotic therapy. Furthermore, cases in which these requirements are not met cannot be included in any group study reported by the E.O.R.T.C. Gnotobiotic Project Group, since information would be insufficient to assure comparable conditions necessary for evaluation. On the other hand, acceptance of these criteria does not prevent a unit from using more extensive procedures where circumstances, personnel, and equipment make these possible and desirable. It is the hope of the Gnotobiotic Project Group that this protocol will assist the various units by providing workable procedures and the information needed to judge the efficiency of human isolation systems.

2. OBJECTS OF THE STUDY

2.1. *To investigate the validity of the use of an isolation system, to protect patients who are highly susceptible to infections*

2.1.1. The efficiency of the various isolation systems will be expressed by the frequency of colonization of the patient by "exogenous"* pathogenic or potentially pathogenic micro-organisms during the period of isolation.

2.1.2. The effect of isolation on the course of the disease in patients with a low defence capacity, will be expressed by the number of days with exogenous* infections† in patients from groups A and B in comparison to group C (see 4).

2.1.3. By investigation of the frequency of infections caused by "endogenous"‡ micro-organisms, information will be acquired about the possible benefit of antibiotic decontamination for the patients with low defence capacity.

2.1.4. The overall number of days with infections will be compared in three groups (see 4).

2.2 *To evaluate and compare the efficiency of systemic antibiotic treatment as a curative treatment, versus antibiotic decontamination of the digestive tract as a preventive type of treatment*

2.2.1. The efficiency of the two systems of treatment will be expressed by (possible) differences in the number of days with infections (either caused by endogenous and/or exogenous micro-organisms).

2.2.2. The efficiency of the various techniques for antibiotic decontamination in use, will be expressed by the number of samples (feces and throat swabs) that are negative for pathogenic and potentially pathogenic microbes (see Appendix 2).

3. CRITERIA FOR THE SELECTION OF PATIENTS

3.1. *Diagnoses*

Diagnoses or conditions forming an indication for admission to the study are:

(*a*) acute leukaemia (AL);

(*b*) chronic leukaemia with bone marrow deficiency;

(*c*) bone marrow aplasia either idiopathic, or induced by drugs, or irradiation;

(*d*) lymphoreticular malignancies;

(*e*) solid tumours;

(*f*) congenital immunological deficiencies;

(*g*) bone marrow transplantation;

(*h*) organ transplantation;

(*i*) burns.

3.2. *Priority cases*

The study will be confined to patients with Acute Leukaemia (AL). This was decided on the basis of their frequency in the units of the Project Group. Only the number of AL patients was found to be sufficient in all participating units, to provide an investigation period as short as possible (2 years) to obtain significant results and to avoid the influence of changes in chemotherapy for leukaemia.

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*"*Exogenous*" *micro-organisms* are those microbes that have not been detected in the inventory made during the admission phase (before the onset of isolation; see 5.3).

†A "day with infection" is defined as:
each day with:
(*a*) positive blood cultures correlated or not with increased body temperature;
(*b*) clinical signs of local infections confirmed by bacteriological culturing;
(*c*) fever of unknown origin with clinical signs of infections. Afterwards correlation can be sought with one of the virus antigens selected.

‡*Endogenous micro-organisms* are those microbes that have been isolated during the inventory made at admission of the patient (see 5.3).

3.3. *Non-priority cases*

When isolation facilities are not occupied by "priority cases", they can be used for patients with the other diagnoses listed in 3.1.

3.4. *The patients are randomized on the several bases*

3.4.1. Age: three age groups are formed of patients with AL:

(*a*) 2 to 15 years (pediatric patients)

(*b*) 16 to 40 years
(*c*) 41 to 60 years } (possible differences in reaction to infection and chemotherapy)

3.4.2. Presence or absence of infection at admission.

3.4.3. Clinical history of the patient:

(*a*) first induction therapy phase

(*b*) second induction therapy phase

(*c*) more induction therapy phases.

3.5. *Exclusion of cases from the study*

All patients for whom admission to the study was considered but rejected on the basis of the criteria given above or other criteria, should be recorded with specification of the contra-indications.

3.5.1. Psychological and/or psychiatric contra-indications (left to the judgement of the clinician).

3.5.2. Renal failure: patients with a significantly decreased glomerular filtration rate, as indicated by a creatinine clearance of less than 60 ml/min/1·73 m^2 body surface, are excluded from the study.

4. METHOD OF ALLOCATION*

There should be a random allocation of the selected cases to one of the following three groups:

Group A: treatment under conditions as group B, but combined with antibiotic decontamination;

Group B: treatment in isolation either in the ultra-clean ward, or in a system for strict isolation (laminar air flow, plastic isolator);

Group C: treatment on the normal ward under standard hospital conditions.

5. ORGANIZATION OF THE STUDY

The study of each patient will be divided into the following phases.

5.1. *Preparation phase*

During this phase the isolation unit will be prepared for use (see Appendix 1).

5.1.1. *Preparation of the unit for patient occupancy*: since the function of the barrier is to protect the patient from environmental sources of infection, adequate means must be employed to indicate that the barrier has allowed entrance to pathogens and potential pathogens. Obviously, isolation should start with a unit free of such micro-organisms (see Appendix 1). The methods and materials for the initial disinfection or sterilization of the isolation system are given in Appendix 1. A container for liquid nitrogen for storage and transportation of the samples must be available or otherwise ordered from the "typing centre" (see Appendix 4).

5.2. *Screening phase*

In this phase a kidney function test (3.6) is to be performed.

The diagnosis, the outcome of this test, the age group to which the patient belongs and the remission-induction phase in which the patient is, as well as the presence or absence of infections, are to be reported to the "statistical centre". This centre will then dictate to which of the three groups the patient will belong in the study (4).

5.3. *Admission phase*

In this phase various investigations are to be performed.

5.3.1. A bacteriological and virological inventory is made of pathogenic and potentially pathogenic species belonging to the patient's microflora:

(*a*) for clinical purposes† (to be performed by the local bacteriologist if required by the clinician);

(*b*) for typing—for this purpose two fecal samples of about 1 g each and two oral washings (in pediatric patients: throat swabs) and in case of infections, isolates from infected sites are to be stored in the liquid nitrogen container;

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*Each patient must be informed about the experimental character of his treatment.

†*Antibiograms*—the antibiotic sensitivities of all isolated potential pathogens should be determined for the so-called non-absorbable antibiotics (neomycin, streptomycin, kanamycin, gentamycin, cephalorin, nystatin, bacitracin, and polymyxin‡) in cases where antibiotic decontamination is being considered. If the patient is not to be decontaminated, the antibiograms of isolated potential pathogens need to be done, unless the clinician and bacteriologist after conferring feel that it is not desirable to do so.

‡Or related antibiotics.

(*c*) virological inventory—a sterile serum sample of at least 2 ml is collected and stored at least at −10°C in (accordingly) minimally 2 vials with 1 ml in each until shipment to the virological centre (15).

5.3.2. Physical examination is to be performed to localize infections. Special attention must be given to the mouth, throat, nose and ears. Consultation of the ear-nose-and-throat specialist is left to the judgement of the clinician. Consultation of a dentist is strongly recommended.

5.3.3. Radiological observation: an X-ray of the chest and the jaws must be made (teeth and sinuses).

5.3.4. Haematological observation: haemoglobin concentration, erythrocyte count, reticulocyte count, thrombocyte count, granulocyte count (band and segmented forms) and lymphocyte count must be determined.

5.4. *Isolation phase*

Clinical, radiological and laboratory observations.

5.4.1. Physical examination: routine physical examination is to be performed twice a week. Special attention must be given to palpation of lymph nodes and spleen [possible enlargement in (viral) infections] as well as to the mouth, the teeth, the throat and the ears. Inspection of the mouth and the throat must be repeated twice weekly, e.g. simultaneously with the oral washing (throat swabbing in young children). In patients selected for decontamination it is strongly recommended to obtain an audiogram before and after decontamination of the patient. This is of particular importance when high doses of oliogosaccharide antibiotics are required for decontamination. The rectal temperature should be measured three times a day, and the highest value of these three determinations recorded. The skin should be inspected for exanthema. Consistency of the feces is to be recorded daily (normal formed, soft, watery).

5.4.2. Radiological observations—an X-ray of the chest is to be made every two weeks thereafter. The chest X-rays should be made under identical conditions before and during isolation to make comparison of the photographs easier.

5.4.3. Haematological observations: haemoglobin concentration, percentage of reticulocytes, thrombocyte count and mature granulocyte count, metamyelocytes; band and segmented forms and lymphocyte count must be determined three times a week.

5.4.4. Observations on renal function: (to be performed in all three groups), three times a week a test for proteinuria and a microscopic examination of the urinary sediment should be performed. Serum creatinine to be determined weekly, the creatinine-clearance every 2 weeks.

5.4.5. Bacteriological observations of the patient: the initial bacteriological examinations, mentioned under 5.3.1. (b), should be repeated twice weekly during the entire study. If septicemia is expected and/or the body temperature of the patient rises above 39°C, a blood sample should be taken for culturing. Strains of p.p. species isolated from positive blood cultures or an infectious tissue, should be stored in liquid nitrogen for shipment to the typing centre.

5.4.6. Virological observations: every 2 weeks a *sterile* serum sample of at least 2 ml is to be collected and stored at minimally −10°C in 2 vials with 1 ml. The vials are to be sent to the virological centre (see Appendix 5) (not necessarily frozen during shipment). The consistency of the feces must be recorded daily (normal, formed, soft, watery).

6. TREATMENT

6.1. *Cytostatic treatment*

Cytostatic treatment, e.g. for leukaemia, should be standardized in the Gnotobiotic Project Group. It is recognized however, that this cannot be realized as long as leukaemia treatment is still a research enterprise. Furthermore, any treatment regimen will most probably be given to the limit of toxicity and periods of severe myelosuppression will be observed frequently.

6.2. *Other treatment*

Any other treatment which could influence the frequency and the course of infections, such as systemic antibiotic treatment, immunosuppressive drugs, irradiation, transfusions, antipyretics, surgical procedures etc. should be recorded mentioning the drugs, the doses and the frequency of administration.

6.3. *Decontamination*

The selection of a decontamination procedure is left to the decision of the attending physician. For information about procedures in use, see Appendix 3.

6.4. *Re-contamination*

The selection of a re-contamination procedure is left to the judgement of the attending physician (see Appendix 3).

7. CRITERIA FOR EITHER WITHDRAWAL FROM OR TERMINATION OF THE STUDY

7.1. *The patient is withdrawn from the study* if the duration of treatment has been less than 2 weeks after the allocation to one of the study groups.

7.2. *The study of a patient is terminated* when there is no longer increased susceptibility to infection. When the leukaemia is going into remission and the granulocyte count is above $1500/mm^3$.

7.3. *The study of a patient is terminated* if isolation has to be terminated for any other* (e.g. social, psychological and/or when death is imminent) reason.

8. LATE OBSERVATIONS

In case death occurs within 4 weeks after the termination of the observation of the patient for the study, it must be recorded.

9. STATISTICAL ANALYSIS AND TERMINATION OF THE EVALUATIVE STUDY

The main difficulty of supraregional or supranational research projects is to guarantee similar standards for the investigations carried out at different places.

This means the standardized performance of such a cooperative study, the similar distribution and the recording and reporting of the same characteristics and pecularities.

In this study the following methods have been chosen.

(1) Forms have been developed to record data which also serve as check-lists to standardize strictly the findings to be obtained and the therapeutic assumptions.

(2) Matched pairs are formed concerning the age level, the number of remission induction treatments, the presence or absence of infection as major complications and preferably the individual hospital center.

(3) The randomisation and the formation of matched pairs is done by the statistical center which is not involved in the examination or in the treatment of patients.

(4) The evaluation will be carried out by a sequential testing method (closed design of Armitage) concerning a clearly defined characteristic as it is the survival rate at the time of 4 weeks after termination of the treatment period.

10. PUBLICATIONS AND LECTURES

Nothing shall be published and no lectures shall be given by any of the participants of the Project Group about either the work of the study group, the Central Service Centres or the results of the study, without the written consent of the Project Group. Obviously, each unit is free to publish observations made on their own isolation ward and patients. However, data derived from one of the Service Centres (Statistical Centre, Typing Centre and Virological Centre) are not free for publication. In such publications and lectures the written directions of the Group should be followed. These directions will especially be concerned with the manner in which the names of the participants are given in publications and lectures.

Appendices 1 to 5 can be obtained by request from Dr. D. Van Der Waaij, Radiobiological Institute TNO, Lange Kleiweg, 151, Rijswijk (ZH), The Netherlands.

The Central Service Centres are:

Statistical Centre

Abteilung für Medizinische Statistiek, Dokumentation und Datenverarbeitung, University of Ulm, W. Germany.

Bacteriological Typing Centre

Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands.

Virological Centre

Institut Pasteur du Brabant, Brussels, Belgium.

Parasitological Centre

Institute for Medical Microbiology, St. Radboud Hospital, Nijmegen, The Netherlands.

Persons interested in additional information or desiring to avail themselves of the services of the group, should contact:

Dr. D. M. Dietrich,
President of the Group,
University of Ulm,
Parkstrasse, 10–11,
79 Ulm/Donau,
W. Germany.

*In case death is the reason for termination, puncture aspiration samples (1 for virological culturing and 2 for bacteriological culturing under aerobic and anaerobic conditions) must be collected from the spleen, the blood (10 ml) the liver and/or bone marrow (2 ml). The samples for shipment to the Virological Centre or a local virologist must be stored at $-70°C$. Shipment can be carried out in "dry-ice".

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Further Studies on the Frequency of Constitutional Chromosome Abnormalities in Patients with Malignant Disease

M. L. O'RIORDAN, A. O. LANGLANDS* and D. G. HARNDEN†

Medical Research Council, Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland

Abstract—*The chromosomes in cultured peripheral blood lymphocytes from 3068 patients with cancer have been examined. Twenty patients have been shown to have a constitutional chromosome abnormality. There was no significant variation of chromosome abnormalities between the various cancer sites and the overall incidence of both sex chromosome aneuploidy and balanced autosomal rearrangements was in good accordance with that of general adult population.*

INTRODUCTION

SEVERAL reports [1–3] have now established that there is an increased incidence of leukaemia in individuals with Trisomy 21 (Down's Syndrome). However, apart from these studies and those surveying populations with leukaemia [4, 5] there are no data available on the frequency of constitutional chromosome abnormalities in a population with other types of malignant disease. As a result it is difficult to assess the significance of isolated case reports of the presence of a malignancy in a chromosomally abnormal individual.

In an attempt to obtain data on this problem, we initiated in 1968 a cytogenetic survey of patients with cancer. A preliminary account of this work has already been published [6]. The present communication presents the total data derived from over 3000 patients.

Accepted 23 Dececmber 1971.

*Department of Radiotherapy, Edinburgh Royal Infirmary, and Western General Hospital, Edinburgh.

†Department of Cancer Studies, University of Birmingham, Birmingham.

Study population

The study was carried out on all new admissions to the wards of the Department of Radiotherapy, Western General Hospital, Edinburgh. This Department serves approximately 1·25 million people who live mainly in the South East of Scotland. While severity of illness, type of treatment necessary and other considerations such as age, or distance from the hospital might determine whether a particular patient is admitted or not, it is not felt that these considerations have introduced any particular bias into this study. On the other hand we are aware that the restriction of the study to patients in radiotherapy wards means that some tumours, for example those of the gastrointestinal tract where the main treatment is by surgery, are under-represented.

MATERIAL AND METHODS

Venous blood samples were obtained from 3303 individuals, 1590 males and 1713 females, during the period from 30th January 1968, to 29th January 1971. A single culture was set up from each sample and cultured for

three days using a modification of the technique of Hungerford [7].

The primary aim of this study was to screen a large population of patients for the incidence of chromosome abnormalities which are assumed to be present in all cells of an individual. Therefore the cytogenetic analysis was based initially on the analysis of two cells and details of the techniques employed have already been published [6]. In some individuals where mosaicism was suspected, further chromosome studies were done on cells from skin fibroblast cultures. In one instance (case 11/69) cells were analysed from a direct preparation of bone marrow.

Wherever possible further cytogenetic studies utilising fluorescent dyes to identify the chromosomes involved have been undertaken on the chromosomes of those patients who had been shown to have a constitutional abnormality. The slides were stained with a 0·5% aqueous solution of quinacrine hydrochloride (Atebrin) and viewed by a Leitz Ortholux microscope, using a Ploem vertical illuminator, a BG12 exciting filter and a 510 nm barrier filter [8].

RESULTS

Satisfactory preparations were obtained from 1471 males and 1594 female patients; a total of 3068 individuals (92·8%).

In 238 instances (119 male and 116 female patients) it was not possible to obtain satisfactory preparations for chromosome analysis. Thirty-two (13·5%) of these patients had tumours of the reticulo-endothelial system. This is a disproportionate failure rate for patients with these tumours compared to those of other sites.

The distribution of the sites of malignancy in these patients for whom a satisfactory preparation was obtained is shown in Table 1.

Twenty patients were shown to have a constitutional chromosome aberration. Eight of these patients had an abnormality of the sex chromosome complement and 12 had an abnormality of the autosomes.

The data on the patients with a sex chromosome abnormality is presented in Table 2. Five were found to have an abnormal number of sex chromosomes in all the cells examined: of these two male patients had a 47,XXY complement and 3 females had a 47,XXX chromosome constitution. A further two female patients both had two cell lines one with a 46,XX complement and one with 47 chromosomes with an additional chromosome belonging to Group C. Examination of buccal smears showed no double sex chromatin bodies and there was nothing to suggest a chromosomal abnormality from the clinical findings. Nevertheless the proportion of cells with the extra chromosome was greater than could be attri-

Table 1. *Survey of patients with malignant disease; distribution of malignancies by site in the study population*

Type of malignancy	Males	Females	Total
Bronchus including trachea	591	116	707
Cervix		253	253
Breast	5	505	510
Thyroid	8	28	36
Gastro intestinal tract excluding mouth and oesophagus	14	17	31
Oesophagus	82	96	178
Sarcomata, including bone sarcomata	26	14	40
Leukaemia, reticuloses, myeloma	102	75	177
Mouth; oro-naso-pharynx, salivary glands, paranasal sinuses, larynx, etc.	239	125	364
Central nervous system	36	25	61
Ovary, vagina, uterus, vulva		162	162
Testis	28		28
Kidney, ureter and bladder	240	98	338
Skin, including lip	80	82	162
Miscellaneous	26	6	32
Total malignancies	1477*	1607†	3084
Cultures failed	119	116	235
Total patients	1590*	1713†	3303

*6 male patients with double primary tumours included in two categories.
†10 female patients with double primary tumours included in two categories.

Table 2. Survey of patients with malignant disease; individuals with sex chromosome abnormalities

M.R.C. Registry No.	Sex	Age	Karyotype	Diagnosis
135/69	M	65	47,XXY	(i) Rodent ulcer left temple†. (ii) Squamous carcinoma right bronchus.
284/70	M	49	47,XXY	Anaplastic tumour:—primary site unknown.
13/69	F	52	47,XXX	Poorly differentiated squamous carcinoma uterine cervix (int stage II).
28/69	F	56	47,XXX	Anaplastic squamous carcinoma of tongue.
232/70	F	64	47,XXX	Rodent ulcer right side of nose.
106/68	F	53	46,XX/47,XXX	Undifferentiated carcinoma bronchus.
130/69	F	48	46,XX/47,XXX	Scirrhous carcinoma right breast.
135/68	M	67	*46,X,inv(Yp+q−) familial	Generalized superficial lymphadenopathy. Histologically "most likely to be a lymphoblastic lymphosarcoma though Hodgkin's disease cannot be excluded."

*Karyotype confirmed by fluorescent microscopy.
†Clinical diagnosis only.

Table 3. Survey of patients with malignant disease; individuals with constitutional autosomal abnormalities

M.R.C. Registry No.	Sex	Age	Karyotype	Diagnosis
166/69	M	76	45,XY,D−,D−,t(DqDq)+	(i) Rodent ulcer right side of nose. (ii) Poorly differentiated squamous carcinoma right upper lobe bronchus.
105/68	F	38	*45,XX,13−,14−,t(13q14q)+ familial	Spheroidal cell carcinoma right breast (Int. stage I).
226/68	F	57	*45,XX,13−,14−,t(13q14q)+ familial	Well differentiated lymphocytic lymphosarcoma R. axilla, R. supraclavicular region.
277/69	F	48	*45,XX,13−,14−,t(13q14q)+	Well differentiated adenocarcinoma left ovary. Mentally retarded.
103/69	F	68	45,XX,D−,G−,t(DqGq)+	Carcinoma right bronchus†. Disseminated sclerosis.
263/69	M	43	46,XY,t(Bq−;Bp+)	Oat cell carcinoma left lung.
240/70	M	54	46,XY,t(1?+;Cq−)	Malignant melanoma right scapular region.
227/68	F	71	*46,XX,t(2p−;7p+)	Papillary adenocarcinoma right ovary.
10/69	M	59	46,XY,inv(Cp−q+)	Carcinoma right lung†.
11/69	M	60	‡46,X,mar+/47,XY,mar+ familial	Well differentiated squamous carcinoma mouth (right lower alveolus), pernicious anaemia.
73/68	F	69	46,XX/47,XX,mar+	Poorly differentiated adenocarcinoma right breast (Int. stage III).
90/70	F	69	46,XX/47,XX,mar+	(i) Thecoma right ovary. (ii) Well differentiated adenocarcinoma uterus.

*Karyotype confirmed by fluorescent microscopy.
†Clinical diagnosis only.
‡46,X,mar+ only observed in bone marrow cells.

buted to the ages of the patients [9] and both patients have been considered to be presumptive 46,XX/47,XXX mosaics.

The last patient in this group was a man with a metacentric Y chromosome which has been confirmed by fluorescent studies to be due to a pericentric inversion. This chromosome has been observed in two of the patient's male sibs and in other male relatives in the family.

Details of 12 patients found to have a chromosome abnormality involving the autosomes are summarized in Table 3. With the exception of one female patient (case 277/69) none of the patients showed any physical or mental abnormalities which might have been associated with the cytogenetic findings. Five of the aberrations were Robertsonian translocations. Fluorescent staining of the chromosomes of three female patients with D/D translocations showed that a number 13 and a number 14 chromosome were involved. Three individuals appeared to have balanced reciprocal translocations, one of which was shown by fluorescence studies to involve the short arms of both a number 2 and a number 7 chromosome. One male patient had a pericentric inversion of a Group C chromosome.

Three patients, one male and two females, were found to carry a small additional chromosome. In the male (case 11/69), the supernumerary chromosome appeared to have satellites on one pair of arms. This chromosome was also seen in all the 100 cells examined from a direct bone marrow preparation both in the XO and the XY cell line and in 30 cells analysed from a skin fibroblast culture. This case has already been published in greater detail [10].

The additional chromosomes in the two female patients were also small, metacentric chromosomes and both patients had a mosaic constitution of 46,XX/47,XX,mar+. Seventy-three per cent of the cultured lymphocytes of case 73/68 contained a supernumerary chromosome which appeared to be satellited on both arms. Twenty-five cells were analysed from a skin fibroblast culture and all cells had a 47,XX,mar+ karyotype.

In case 90/70, 26% of lymphocytes had the extra chromosome but it was not possible to obtain a skin biopsy for further study. The extra chromosome in this case was metacentric and no satellites were observed.

DISCUSSION

Ideally the morbidity and mortality patterns associated with chromosome abnormalities should be based on data from individuals identified at birth and followed through life together with suitably matched controls. Clearly this form of study would require not only a large number of persons with aberrant karyotypes but would also require a long time for significant results to be obtained.

Incidence studies on selected populations present their own particular problems and the present investigation has at least two major limitations.

Table 4. Comparison of the incidence of chromosome abnormalities in cancer patients, Edinburgh selected adult and neonatal populations (incidence/1000 individuals).

	Survey of cancer patients	Edinburgh surveys selected adult populations	Edinburgh surveys of babies
Number of individuals	1471 males 1587 females 3068 total	5543 males 1074 females 6617 total	5733 males 1776 females 7529 total
Sex chromosomes			
47,XXY	1·4	3·8	1·4*
47,XYY	0	2·0*	1·7
47,XXX	3·1†	0	1·7
Autosomes Balanced rearrangements	2·9	2·7	1·06
Supernumerary chromosomes	0·91†	0·30†	0·13†

*Figures include 1 mosaic.
†Figures include 2 mosaics.

Firstly, in this study the frequency of the tumours at different sites (Table 1) is not representative of the frequency with which these tumours occur in the general population, but it is determined by the usefulness of radiotherapy in their management. As a result tumours of the gastro-intestinal tract and leukaemias are underrepresented. Secondly, the analysis of only two cells from each patient in the survey in the first instance means that it is likely that some individuals with a mosaic constitution who have a normal cell line will not be detected.

There are only limited data on the incidence of karyotype anomalies in the adult "general population" to compare with results from this study. Although a large amount of information has come from cytogenetic surveys of new born infants, care must be taken in comparing those results with surveys of adults as it is known that certain chromosome abnormalities are associated with increased childhood mortality.

Table 4 summarizes the incidence of various sex chromosome and autosome abnormalities found in this survey, previous and current Edinburgh neonatal surveys [11] and selected adult populations [12]. These adults were considered to be representative of the general population at large and institutionalized mentally subnormal individuals were excluded. No significant difference has been shown between the three populations in the incidence of the more common types of sex chromosome aneuploidy. The incidence of 3·1/1000 of 47,XXX and 46,XX/47,XXX females in the cancer patients is slightly higher but this is not a statistically significant increase ($P < 0{\cdot}1$). Individuals with pericentric inversions of the Y chromosome are relatively uncommon but the finding of one male patient with this abnormality would accord with the estimated frequency by Smith and Jacobs [12] of 0·58/1000 individuals in the combined neonatal and adult population.

There is a marked discrepancy between the frequency of balanced structural heterozygotes in the two adult populations compared to the incidence found in new born infants ($0{\cdot}01 < P < 0{\cdot}02$). Jacobs [12] has discussed in detail the various explanations which might account for these differences. However, since there appears to be no appreciable difference in the incidence of balanced rearrangements between the general adult and the cancer patient populations, it would not seem from our data that persons who carry such abnormalities are predisposed to the development of malignancy.

The finding of three individuals with a supernumerary chromosome does not represent a significant increase over either the figures derived from the study of neonates ($P < 0{\cdot}05$) nor those found in the general adult population ($P < 0{\cdot}07$). Supernumerary chromosomes are more generally found in individuals who are mentally subnormal and who may also have congenital abnormalities but none of the three patients identified in this survey showed any physical or mental defects which could be attributed to the presence of the extra chromosome. Family data has been obtainable only in case 11/69. Chromosome studies were done on the only child, a son, of this patient, and the supernumerary chromosome was not observed. However, both the sister of the propositus and her daughter were shown to have a 47,XX, mar+ karyotype. It has not been possible to obtain any information on the transmission of the extra chromosome for either of the two female patients.

With the exception of the studies on Down's Syndrome and leukaemia [1–3], information on the frequency of constitutional chromosome abnormalities has been limited to populations of non-mongoloid leukaemic children and male patients with carcinoma of the breast.

Borges *et al.* [4] studied a series of non-mongoloid children with acute leukaemia in remission. They studied 25 children and found three with abnormal karyotypes; a 47,XXY male, a 47,XYY male and a 47,XY,?F+male. Following this report, Zuelzer and Cox [5] did a larger serial study of 200 leukaemic children in remission but they claim to have found 4 children with an apparent constitutional chromosome abnormality. The difference of incidence in these two studies is difficult to explain but the higher frequency in the smaller survey [4] may perhaps be a chance finding.

Two reports have demonstrated an increased frequency of breast carcinoma in males with Klinefelter's syndrome, [14, 15]. Gynaecomastia can be a feature of this syndrome and the increase in incidence of breast carcinoma may be a secondary reflection of a hormonal disturbance rather than a direct consequence of the individual's abnormal sex chromosome complement.

From the results of this survey, valid statistical comparison of the frequency of chromosome abnormalities according to the site of the disease is not possible due to the wide variation in the number of individuals with cancer of specific sites. However, it does not appear that there is any concentration of karyotypically abnormal individuals in any one tumour group.

Three of the patients with abnormal karyotypes had more than one primary tumour but in two instances the second tumour was a rodent ulcer and it is not considered that this represents an abnormal incidence of multiple primary tumour in these patients.

In our preliminary report on this study the possibility was raised that there might be an excess of chromosomally abnormal female patients with breast carcinomas and in whom the disease occurred at an early age. This has not been confirmed after the study of a further 328 women with breast cancer in the main series. In addition a population of women in whom a diagnosis of breast cancer had been made before the age of 40 is being examined. So far 59 such cases have been studied and all have been found to have normal chromosomes.

Within the group of female gynaecological tumours, thirty-four patients had ovarian tumours. Of these, two individuals had autosomal translocations and one person had a mosaic constitution with one cell line containing an extra, small chromosome. Again the small numbers studied make it impossible to assess whether or not this is a real increase but further individuals with ovarian tumours are currently being investigated.

There have been isolated reports [16] of so-called "cancer families". Where cytogenetic studies have been done, the majority of affected individuals have had a normal karyotype but in a few cases a familial chromosome abnormality has been implicated as a cause of the increased incidence of malignancy in the family or sibship. Perhaps the most notable of these reports has been the apparent association between chronic lymphatic leukaemia and a deletion of the short arms of a chromosome number 22 (Ch^1 chromosome) in one family in New Zealand [17, 18]. However, no other examples of this particular association have been reported.

A limited part of this study was to investigate the families in which a chromosomally abnormal propositus had been identified. As a result a further 21 individuals with a similar chromosome abnormality to the propositus, have been identified from four families. None of these persons had or has subsequently developed a tumour but it should be noted however that a proportion of these relatives were young. In addition a further 8 women from families with a high incidence of breast carcinoma have been examined and none have been found to have a chromosome abnormality.

The results from this present cytogenetic survey do not form a definitive study on the role, if any, of constitutional chromosome abnormalities in malignant disease. The example of Down's syndrome and an increased risk of the development of leukaemia remains unique. However, until more refined data can be collected, our results indicate that there is not an increased susceptibility to cancer in general among persons who carry autosomal rearrangements or the more common types of sex chromosome aneuploidy.

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The Relation Between Sebum Production and Plasma 17-Oxosteroid Levels in Normal Women and in Patients with Breast Cancer

D. Y. WANG, R. D. BULBROOK, J. GUILLEBAUD and A. LEWIS

Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, and The Luton and Dunstable Hospital, Luton, Beds.

Abstract—*The plasma concentration of dehydroepiandrosterone sulphate and sebum production have been determined in patients with breast cancer and normal women. Breast cancer patients generally had higher levels of sebum production than normal women. The higher level of sebum production in women with breast cancer was not associated with high amounts of plasma oxosteroids.*

THERE is much evidence to suggest that the activity of the sebaceous glands is largely controlled by the androgens [1, 2, 3] and Pochi and Strauss have shown that testosterone, androstenedione or dehydroepiandrosterone administered to adult males stimulates sebum production [4]. They have also reported that the sebum production of women with breast cancer is significantly increased above that of normal women, or of women with other cancers [5]. More recently, Burton *et al.* have confirmed that sebum production in women with breast cancer is higher than in normal women [6]. This is a puzzling finding since other workers have found a low excretion of urinary androsterone and aetiocholanolone in patients with both early and advanced breast cancer [7, 8, 9, 10]. Deshpande *et al.* [11] and Wang [12] reported that plasma 17-oxosteroids were generally within or at the lower end of the normal range and Wang *et al.* [13] found normal levels of plasma testosterone in such patients. From these results, it might have been expected that sebum production in patients with breast cancer would be low or normal, rather than high. For this reason, sebum production and levels of dehydroepiandrosterone sulphate (DS) in the peripheral blood were measured simultaneously in normal women and in patients with breast cancer.

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METHODS

The patients with breast cancer were being treated at the Luton and Dunstable Hospital, England. "Early" cases (16 patients) were in Stage I or II (Manchester); following the T N M system these are cases designated from T1 N0 M0 up to T2 N1 M0. "Advanced" cases (19 patients) are defined as those more advanced either locally (Stage III, up to T4 N3 M0) or with distant metastases (Stage IV, and combination of T and N symbols including M). In every case the diagnosis was confirmed histologically.

The normal women investigated were 20 volunteers from the staff in the same hospital or at Edgebury Convalescent Hospital, Woburn Sands. At the time of collection neither cancer patients nor controls were receiving any steroid therapy. However, three cancer patients have had androgen therapy. In two of these, this treatment had been terminated 3 years before this study, and in the other, 3 months.

Method of sebum collection

The back of each subject was washed three times with cotton wool swabs soaked in acetone, and this was followed by a dry swab. Three thicknesses of absorbent paper (S.C.F. white paper, 13g/m^2, J. R. Crompton and Bros. Ltd., Bury, Lancs) were then applied closely to the area, held in place and sealed by a slightly larger sheet of polythene whose edges were stuck to the skin by strips of "Micropore" or "clear-tape". The area of each paper sheet was 835 cm^2.

The subject was instructed to sit in one position, leaning back on her pillows or the back of an armchair, for 3 hr. After removal the three sheets were stored in re-distilled acetone. The papers were subsequently extracted continuously with acetone (250 ml) in a Soxhlet extractor for 3 hr. The acetone was taken to dryness and the residue was dissolved in hexane. The hexane extract was filtered through celite, taken to dryness and the residue weighed. After 24 hr *in vacuo* the residue was re-weighed. The two weighings rarely differed by more than 0·3 mg. When they did, a further 24 hr drying *in vacuo* generally led to an agreement between the weighings (not more than 0·3 mg difference). Blank paper taken through this procedure gave a residue weight of 0·8 mg.

Blood (25 ml) was taken from an antecubital vein during the time the sebum was being collected. It was heparinized, the plasma was separated and stored at −20°C. Plasma dehydroepiandrosterone sulphate was determined by the method of Wang *et al.* [14].

RESULTS

Sebum production

There is a significant decrease in the amount of sebum production with increasing age in normal women, and in patients with either early or advanced breast cancer (Fig. 1). The majority of the values in both groups of cancer patients are higher than those for the normal controls, at all ages. If the values are age-corrected, the average level of sebum production in patients with advanced breast cancer is significantly higher than that of the controls (31·2 mg and 20·4 mg respectively; $F = 6{\cdot}06$, $P < 0{\cdot}025$). Sebum production by patients with early breast cancer is also higher than that of controls (age-corrected means 31·69 mg and 22·3 mg respectively) and the difference is significant ($F = 6{\cdot}56$, $P < 0{\cdot}025$). There was no significant difference between the sebum production of early and advanced breast cancer

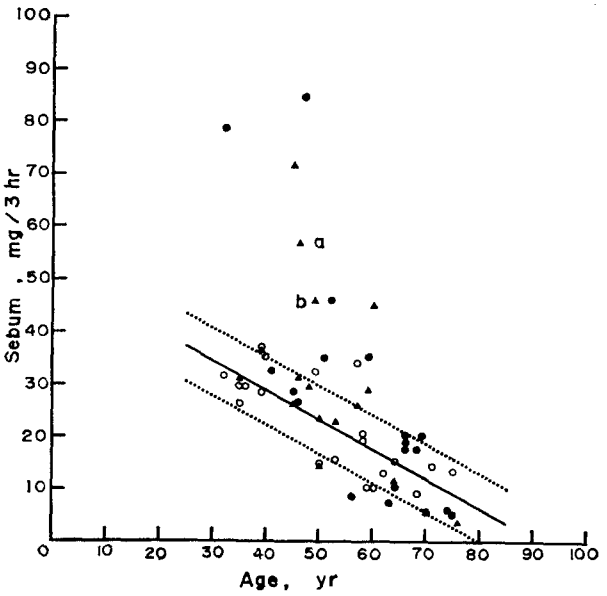

Fig. 1. The symbols are, ○ for control women, ▲ and ● for women with early or advanced breast cancer, respectively. A and B refer to women with another primary tumour in the contralateral breast. The line is the regression line for normal women together with the 95% confidence limits. The equation of this line is $y = 50{\cdot}76 - 0{\cdot}551x$, where y is sebum production expressed as mg/3 hr. and x is age in years. The level of significance is $P < 0{\cdot}001$. The equations and levels of significance of the early and advanced cancer groups are:
$y = 73{\cdot}21 - 0.805x$ ($P = 0{\cdot}05$) and
$y = 107{\cdot}02 - 1{\cdot}378x$ ($P < 0{\cdot}02$), *respectively.*

subjects. Two of the patients with early breast cancer (marked A and B in Fig. 1) produced excessive amounts of sebum, the amounts being approximately double those found in normal women of the same age. These patients both had a second primary growth in the contralateral breast (judged on histological evidence and the fact that there were no other clinical signs of metastases).

Plasma DS levels

There is a significant decrease in plasma DS concentration with advancing age in normal women and patients with advanced breast cancer (Fig. 2). There was no marked difference in the average concentration of DS in the three groups of women but the values found for patients with advanced breast cancer tended to lie at the lower end of the normal range.

Relation between sebum production and plasma DS concentrations

To examine the relationship between sebum production and plasma DS levels, these parameters have been expressed as a ratio (Table 1). The average of this ratio in normal women is half that found in women with either early or advanced breast cancer, but are only signifi-

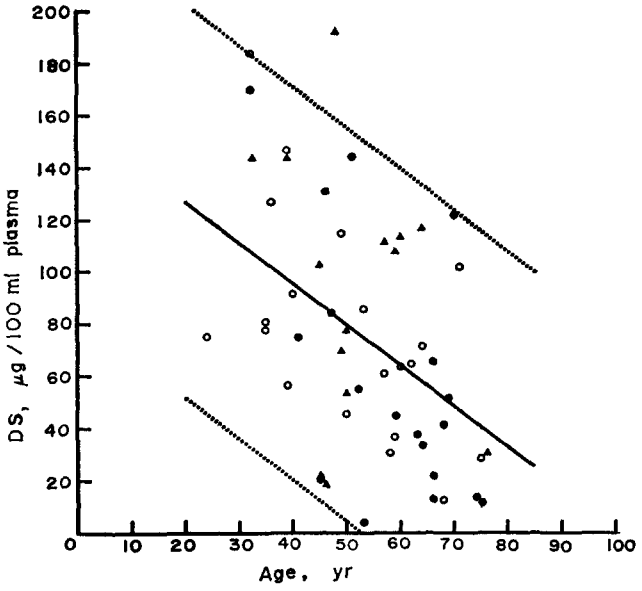

Fig. 2. Relationship between plasma levels of dehydroepiandrosterone sulphate and age. The symbols are, ○ for control women, ▲ and ● for women with early or advanced breast cancer, respectively. The line is the regression line for normal women together with the 95% confidence limits. The equation of this line is $y = 157{\cdot}88 - 1{\cdot}586x$, *where y is plasma DS expressed as μg of free steroid per 100 ml plasma and x is age in years. The level of significance is* $P < 0{\cdot}02$. *Similar data for the early and advanced cancer groups are:*

$y = 152{\cdot}15 - 1{\cdot}444x$ (P = *NS*) *and*

$y = 191{\cdot}14 - 2{\cdot}249x$ ($P < 0{\cdot}02$), *respectively.*

Table 1. *Ratio of sebum production and plasma DS concentration*

	Normal women	Early breast cancer	Advanced breast cancer
Mean	0·344	0·686	0·655
S.D.	0·182	1·041	0·547
n	19	14	19
t	(cancer *v* normal)	1·41	2·35
P	—	N.S.	< 0·05
Differences between variances			
F	(cancer *v* normal)	33·3	9·01
P	—	< 0·001	< 0·001

Ratio is expressed as sebum production (mg per 3 hr) to DS concentration (μg per 100 ml plasma). The number of observations is denoted by *n*.

cantly different from normals in the advanced disease. However, there is a considerable increase in the variance of the results in both cancer groups (Table 1) due to high ratios in some of the cancer patients.

In women with early breast cancer 21% (3 out of 14) had ratios above the upper 95% normal limit and 37% (7 out of 19) with the advanced disease had values above this limit.

DISCUSSION

The results of this study show that the sebum production of pre- or post-menopausal women with cancer of the breast is higher than that of normal women. This finding confirms that of Krant *et al.* [5] and Burton *et al.* [6] who found that sebum production was raised in post-menopausal women with breast cancer.

Our interpretation of the results is somewhat different from that of Krant and his colleagues who suggested that women with advancing breast cancer have an androgen-stimulated environment [5]. In terms of DS levels, our own findings do not support this theory since, on average, patients with breast cancer produce larger quantities of sebum at a given plasma level of DS than do normal women.

An excessive androgen production seems an unlikely explanation for the raised secretion of sebum. The majority of reports indicate that the urinary excretion of the androgen metabolites is lower than normal in both early and advanced breast cancer patients. Plasma total 17-oxosteroids are normal or at the lower end of the normal range. Even in the present small-scale study, about a third of the patients have low peripheral plasma levels of DS.

Wang *et al.* [14] found normal levels of plasma testosterone in patients with breast cancer. Also Strauss *et al.* [15] failed to find a correlation between amounts of testosterone in the plasma and sebum production. The results imply that excessive sebaceous secretion is not due to high plasma testosterone concentrations. However, the possibility that excessive sebum production in women with breast cancer is due to an increased end-organ sensitivity cannot be excluded.

Oestrogens administered in pharmacological amounts have been shown to inhibit sebum production in both laboratory animals and in man [3, 16]. Whether breast cancer patients have increased levels of oestrogen is not known although there is evidence that the excretion of urinary oestriol is reduced (see Lemon [17]).

Sebum production appears also to be controlled by pituitary sebotrophic factors, [18, 19, 20, 21] TSH, ACTH and β-lipotrophin [22, 23, 24]. Ebling *et al.* [25] have shown that in the rat, testosterone is totally inactive in stimulating sebum production in the absence of the pituitary gland. Activity of testosterone can be restored by administering either prolactin or growth hormone. These results imply a permissive role for the pituitary gland but a synergistic action between androgens and pituitary hormones has not been ruled out. A pituitary factor (or

factors) appears to be involved in the response of breast cancer patients to treatment [26].

It is clear that sebum production is controlled by many hormonal factors and that a further investigation is warranted of sebum production in patients with breast cancer in which the principal plasma androgens and oestrogens were measured, and in which growth hormone (and possibly prolactin) are also determined.

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Clinical Trial of Nafoxidine, an Oestrogen Antagonist in Advanced Breast Cancer*†

E.O.R.T.C. BREAST CANCER GROUP‡

Abstract—*An EORTC type I clinical study of the oestrogen antagonist nafoxidine was performed in patients with advanced breast cancer, according to the protocol of the European Breast Cancer Group (EORTC). Eight objective remissions were observed out of 23 evaluable cases (35%). The drug was well tolerated; marked photo-sensitivity of the skin to sunlight was the only important side-effect.*

INTRODUCTION

NAFOXIDINE hydrochloride§ (U–11, 100A), a diphenyldihydronaphtalene derivative, is an oestrogen antagonist. It inhibits the oestradiol-induced increase of uterine weight in oophorectomized animals, although it has a weak oestrogenic action of its own when administered alone [1]. It prevents oestradiol retention in the uterus, both *in vivo* and *in vitro* [2, 3]. Like other non-steroidal anti-oestrogens [4], nafoxidine seems to produce this effect by competing effectively with oestradiol for its specific uterine receptors [5].

This compound also prevents oestradiol retention *in vitro* in the DMBA-induced, hormone-dependent rat mammary carcinoma [3] and in human breast cancers [6]. Moreover, it has anti-tumour effects on the rat mammary carcinoma: it prevents tumour induction [7] and inhibits tumour growth [7, 8].

The purpose of this trial was to assess the therapeutic activity of nafoxidine in advanced breast cancer.

MATERIAL AND METHODS

The criteria used for selection of patients and evaluation of results are those described in the protocol of the clinical trials of the Group [9], with minor modifications.

The main feature of the protocol are biopsy proof of the disease, post-menopausal status of the patients, use of objective criteria of remission, evaluation of the results by two non-participating observers.

Nafoxidine‖ was administered orally at a dose of 60 mg 3 times a day for at least 3 months, unless conspicuous progression occurred in the interval. The treatment was continued indefinitely in cases of objective remission.

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†The manuscript was prepared by J. C. Heuson, A. Coune, and M. Staquet (Service de Médecine, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, Brussels, Belgium).

‡This group is one of the co-operative groups of the European Organization for Research on Treatment of Cancer (EORTC). Chairman: J. C. Heuson; Secretary: A. Coune; Statistician: G. Vamecq; Representative of the EORTC Council: H. J. Tagnon; Co-ordinator: M. Staquet.

Participants to this study are: *Akademisch Ziekenhuis Sint-Rafaël, Leuven, Belgium* A. Drochmans, J. Wildiers. *Antoni van Leeuwenhoek-Huis, Amsterdam, The Netherlands* E. Engelsman. *Institut Jules Bordet, Brussels, Belgium* A. Coune, J. C. Heuson, J. Michel, M. Staquet, H. J. Tagnon. *Rotterdamsch Radio-Therapeutisch Instituut, Rotterdam, The Netherlands* H. van Gilse, D. Blonk-Van der Wijst.

§Nafoxidine hydrochloride = 1 – {2 – [*p* – (3,4-dihydro-6-methoxy-2-phenyl-l-naphtyl) phenoxy]-ethyl} -pyrrolidine hydrochloride.

‖Nafoxidine hydrochloride was supplied as 30 mg tablets by the Upjohn Co., Kalamazoo, Mich., U.S.A. through the courtesy of Dr. S. Carter, National Cancer Institute, Bethesda, Md., U.S.A.

RESULTS

Table 1 gives the number of patients evaluated, their category distribution and the number of objective remissions. Twenty-three patients were treated for at least 14 days. Eight objective remissions were observed (35 %; 95 % confidence limit: 16–57 %). No lens opacification attributable to the administration of the compound was detected. No weight gain and no significant elevation of the blood pressure were recorded.

Table 1. Clinical trial with nafoxidine (U−11, 100A) in advanced breast cancer. Distribution of patients and results.

Dominant lesion	Years after menopause				Total
	< 1	1–4	5–9	> 10	
Soft tissue	—	1/1	1/2	2/3	4/6
Osseous	0/1	—	0/2	3/8	3/11
Visceral	—	—	0/1	1/5	1/6
Total	0/1	1/1	1/5	1/16	8/23

The figures in the table give the number of objective remissions over the number of cases treated.

No effect on hematopoiesis and no modification of liver function tests were noted. Hypercalcemia developed at the beginning of the treatment in one patient with metastatic bone involvement and led to its interruption; this patient is not included in the present study. Alopecia occurred in one patient and mild ichthyosis of the skin in another. Marked photosensitivity was observed in 13 of the 23 evaluated patients. It consisted of a skin erythema on direct or even indirect exposure to sunlight during summer. It was never complicated by burns.

In 14 patients, vaginal cytology was carried out at the beginning and at the end of the treatment. In 11 cases, the vaginal smears revealed a transformation from an atrophic or non-specific picture to an oestrogenic picture.

DISCUSSION

This clinical trial indicated that nafoxidine is effective in patients with advanced breast cancer. The remission rate (35 %) is of the same order as that achieved by the major endocrine ablative procedures, although the number of patients in this series is too small to draw conclusions as to the relative efficacy of the drug compared to conventional endocrine treatments. On the other hand, side-effects were mild and did not lead to discontinuation of therapy, except in one case of hypercalcemia where the treatment was tentatively incriminated. Photosensitivity of the skin to sunlight was a frequent occurrence during summer-time and presented itself as a transient erythema without burns.

The mechanism of induction of remissions by nafoxidine is unclear. As judged by the picture of oestrogenic stimulation of the vaginal smears observed in most of our patients during treatment, nafoxidine appears to act more as an oestrogen than as an anti-oestrogen in post-menopausal women. Like other non-steroidal anti-oestrogens, nafoxidine is known to be weakly oestrogenic and to bind to the oestrogen receptors in the normal target tissue [4, 5]. In view of the recent finding that in one third of all cases metastatic breast cancer tissue also contains oestrogen receptors [6], it is tempting to speculate that nafoxidine binds to these receptors and thereby produces inhibitory effects of an unknown nature on the cancer cells.

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Action of 1-β-D-Arabinofuranosylcytosine on Mammalian Tumor Cells—1. Incorporation Into DNA

RUDOLF K. ZAHN, WERNER E. G. MÜLLER, WOLF FORSTER, ARMIN MAIDHOF and RUDOLF BEYER

Institut für Physiologische Chemie, Johannes Gutenberg Universität, 65 *Mainz, Johann Joachim Becher Weg* 13, *West Germany*

Abstract—*Synchronized mouse lymphoma cells were treated with ara-C-^3H at the beginning of the S-phase with twice the ED_{50} concentration. In DNA isolated from these cells ara-C could be traced. It could be shown that the radioactivity in nucleotide- and nucleoside fractions obtained after enzymic digestion of ara-C-DNA was due for more than 87% to ara-C-^3H. The radioactivity of ara-C-^3H can be almost quantitatively attributed to cytosine and arabinose. An incorporation of ara-C into RNA could not be detected.*

INTRODUCTION

1-β-D-ARABINOFURANOSYLCYTOSINE (ara-C) can inhibit cell proliferation and DNA synthesis of mammalian cells [1, 2] and of DNA viruses [3]. Two reports have shown that ara-C is able to inhibit proliferation of malignant tumors [4, 5]. About the main target of ara-C different views exist [survey: 6]. In one hypothesis [1] it is suggested that the incorporation of ara-C into DNA is the cause for the inhibition of DNA synthesis and thus for the inhibition of tumors. Data on the *in vivo* incorporation of ara-C into the DNA of mammalian cells have been given by several authors [7, 8, 1]. *In vitro* incorporation of ara-C into DNA by a DNA-synthesizing system has been described [9]. Up to now the results indicating an incorporation of ara-C into DNA cannot be considered as definitive evidence in the light of the cautions stressed by Cohen [10].

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The abbreviations used are: ara-C: 1-β-D-arabinofuranosylcytosine; ara-C-DNA: ara-C-^3H-labeled DNA; DNase: deoxyribonuclease; dCyd: deoxyribosylcytosine; Cyt: cytosine.

In this paper the question whether ara-C molecules as such are incorporated *in vivo* into the cellular DNA during the DNA synthesis is therefore studied again. The experiments were performed with intact mouse lymphoma cells. It has been definitively established that ara-C-^3H is incorporated into the nucleotide sequences of cellular DNA. It was possible to prove that D-arabinose is associated with the labeled cytosine in the nucleoside- and nucleotide fractions.

MATERIAL AND METHODS

1. *Sources of materials*

The following materials were obtained: Unlabeled ara-C, D-ribose, deoxy-D-ribose and D-arabinose from H. Mack, Illertissen; caesium chloride, anilinephthalate from E. Merck, Darmstadt; insta-gel from Packard Instrument, Zürich; Sephadex G-25 from Deutsche Pharmacia, Frankfurt; thin layer plates (MN-polygramm, cel 300) from Macherey and Nagel, Düren; ara-C-^3H labeled generally from Schwarz Bioresearch, Orangeburg (The

ara-C-^{3}H has been purified by thin layer chromatography in butanol-NH_4OH, prior to use described later. The ara-C-^{3}H, 12 μmole per ml, had a specific activity of 1·1 Ci per mmole); DNase I (2000 units/mg) and phosphodiesterase (venom, potency = 0·3–0·2% AMP/0·25 mg) from Worthington, Freehold; alkaline phosphatase (calf intestine, activity = 2500 μmole of phosphate liberated per mg per 30 min, 37°C, pH 9·0) from Mann, New York.

2. *Cells*

L5178y cells [11] were grown in Fischer's medium for leukemic cells from mice (Grand Island Biological Comp., Grand Island) in suspension cultures [12]. In a batch of 250 ml cells were inoculated at a concentration of 6×10^3/ml and incubated at 37°C in roller tubes. After 120 hr the cells reached the stationary phase and are, according to the data given by Tobey *et al.* [13], partially synchronized. The cells were transferred by centrifugation (800 × **g**, 20°C, 3 min) into 420 ml of fresh medium, containing 2·5 mM thymidine. After 17 hr 80% of the cells were at the beginning of the S-phase. These cells after transfer into thymidine-free medium, containing purified 53 ng ara-C-^{3}H/ml with a specific activity of 1·1 Ci/mmole were incubated; during 24 hr the cells run through a complete cell cycle. The cells were washed three times with 0·07 M K-phosphate buffer (pH 7·2) by centrifugation.

3. *Extraction of DNA*

DNA from the cells was extracted by the method of Kirby [14]. The ratios of optical densities of the following wavelengths (mμ) were 260 : 280 = 2·17, 260 : 240 = 1·58 and 260 : 230 = 1·93.

4. *Digestion of ara-C-DNA*

The enzymic digestion of the DNA was performed in a pH-stat (Dosimat, Metrohm, Herisau). First the DNA was degraded by DNase I under limiting substrate conditions at 37°C and pH 7·0. The reaction mixture contained: 1·0 ml DNA solution (usually 200 μg/ml) 0·1 ml 0·1 M $MgCl_2$ and 0·1 ml DNase I (1 mg/ml). The reaction was terminated when the enzyme reaction stopped, usually after 30 min. The resulting DNA fragments were further hydrolyzed by the addition of phosphodiesterase and alkaline phosphatase at 37°C and pH 9·0 for 90 min. The reaction mixture contained the following components: 1·0 ml DNA products resulting from DNase I digestion, 0·1 ml 0·1 M $MgCl_2$, 0·15 ml phosphodiesterase (1 mg/ml) and 0·1 ml alkaline phosphatase (1 mg/ml).

For a complete hydrolysis the DNA fragments obtained by enzymic treatment of ara-C-DNA were incubated in 2 N KOH at 100°C for 2 hr. The hydrolysate was neutralized with conc. $HClO_4$ and the resulting $KClO_4$ removed by centrifugation.

5. *Analytical methods*

DNA was determined by the method of Kissane *et al.* [15], pentose in solution by the orcinol reaction described by I-San Lin *et al.* [16], pentose on thin layer plates with aniline-phthalate [17]. The nucleotides, nucleosides and bases were located in ultra-violet light (Mineralight-u.v. lamp). Thin layer chromatography was performed in an ascending system on cellulose plates with water saturated *n*-butanol-15 N NH_4OH solvent (100 : 1) [18]. In this solvent system the different R_f values are: ara-C 0·32, dCyd 0·40, Cyt 0·38, D-arabinose 0·19, D-ribose 0·31 and deoxy-D-ribose 0·45. For spotting the radioactivity, the developed chromatograms were cut into pieces (0·5 cm by 1·0 cm). Each strip was eluted with 0·4 ml of 0·1 N HCl. For determination of radioactivity samples of 0·05–0·2 ml were dissolved in 10 ml of insta-gel and counted in a scintillation counter (TriCarb Packard Inst., La Grange). Preparative centrifugation was performed at 20°C in a 40·3 rotor of the Spinco model L2-65 for 90 hr at 30 000 rev/min. The initial density of the CsCl was adjusted to 1·700 g/cm^3; CsCl was dissolved in 15 mM tris (pH 8·0). Gradients were analyzed by collecting 25 fractions by displacement with saturated CsCl solution. The optical density was measured in a Zeiss PMQ II spectrophotometer. For concentrating of DNA, the samples were sedimented at 2°C in 0·1 M CsCl solution in a 40·3 rotor at 30 000 rev/min for 12 hr.

RESULTS

To synchronized mouse lymphoma cells (L5178y) ara-C was added 0·5 hr before entering the S-phase. 53 ng ara-C per ml were added; this concentration is twice the ED_{50} concentration (ED_{50} is the concentration which induces in a dose-response experiment a 50% inhibition of cell proliferation). After a complete cell cycle cells were harvested and the DNA extracted. From $1·4 \times 10^8$ cells 810 μg of DNA could be obtained on the average. This corresponds to a yield of about 85% (DNA content per lymphocyte: $6·8 \times 10^{-12}$ g; [19]). The

specific activity for ara-C incorporation was $5{\cdot}05 \times 10^{-4}$ moles ara-C per mole DNA nucleotide. By this it can be calculated that about 2000 DNA nucleotides correspond to one molecule of ara-C.

From the resulting DNA preparation the DNA was chromatographically separated on a Sephadex G-25 column (diameter: 6·8 mm; height: 77·5 cm; elution with 0·01 N NH_4HCO_3) from oligonucleotides and other low molecular DNA components. The DNA fraction (Kd: 0–0·068) was subsequently purified from RNA residues by CsCl density-gradient-centrifugation. The banding profile of the ara-C-DNA in the CsCl gradient is shown in Fig. 1. A buoyant density of the ara-C-DNA of 1·7025 (g/cm^{-3}) is found. The total radioactivity accompanies the DNA (range of buoyant densities from 1·67 to 1·75). This indicates that the radioactivity may be associated with

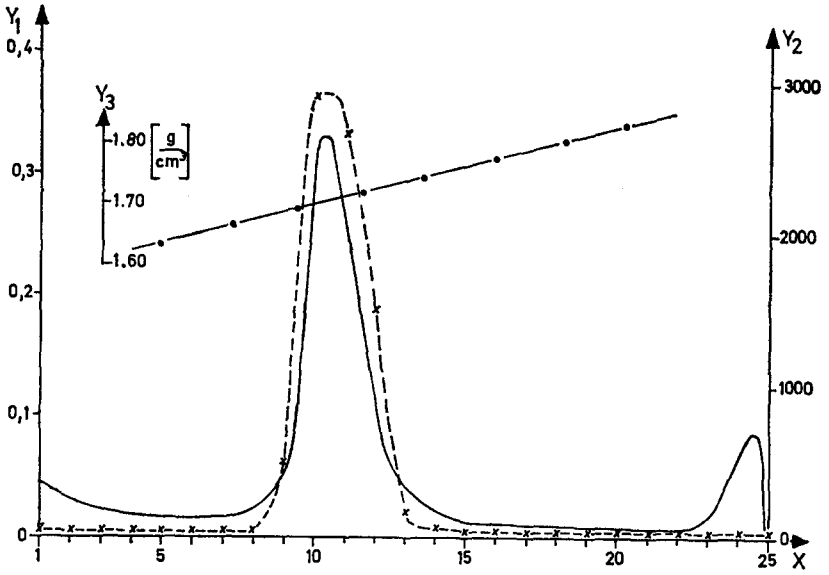

Fig. 1. Buoyant density of ara-C-DNA. 50 µl DNA isolated from ara-C-^3H incubated L5178y cells, were transferred into a CsCl solution and centrifuged (see under methods). 25 fractions (0·4 ml) were collected and buoyant density (·—·) and optical density (—) were determined. For radioactivity (x - - x) 50 µl aliquots were counted in a scintillation counter (see under Methods).

x-axis: number of the fractions; y_1-axis: optical density at 260 mµ; y_2-axis: radioactivity, in dpm/50 µl; y_3-axis: buoyant density in g/cm³.

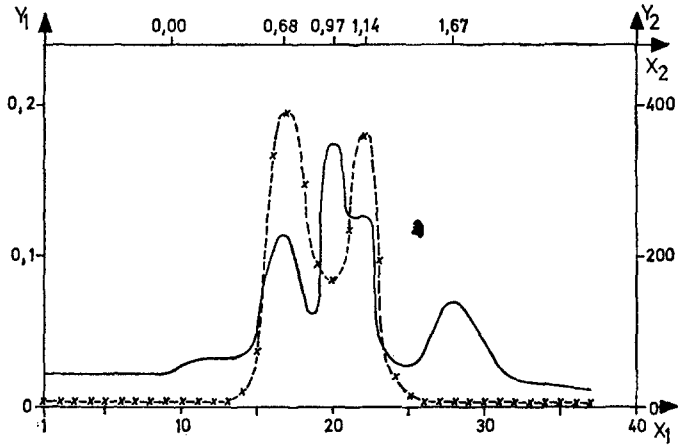

Fig. 2. Chromatographic separation of DNA fragments, digested by DNase I, phosphodiesterase and alkaline phosphatase on Sephadex G-25 (as described uder methods). 0·5 ml of the hydrolysate corresponding to 25 µg ara-C-DNA have been fractionated. Column: 12 mm × 81·7 cm; volume per fraction: 4 ml. Elution was performed with distilled water.

x_1-axis: fraction number; x_2-axis: R_f-values, pyrimidine-nucleotides: 0·68, purine nucleotides: 0·97, pyrimidine-nucleosides: 1·14, purine-nucleosides 1·67. y_1-axis: optical density at 260 nm (—); y_2-axis: radioactivity (x - -) (dpm per 50 µl).

the DNA. The RNA fraction (buoyant densities greater than 1·8) is free from radioactivity.

The DNA fraction (density from 1·67–1·75 $g \times cm^{-3}$) was pooled and concentrated by sedimentation. Subsequently the DNA was digested enzymically with DNase I, phosphodiesterase and alkaline phosphatase. The hydrolysate when fractionated on Sephadex G-25 (Fig. 2), shows two symmetric peaks of radioactivity, one in the pyrimidine nucleotide and one in the nucleoside fraction. The volume of these two fractions was reduced by evaporation in vacuo at 20°C. Samples of 50 µl, containing 870 ±80 dpm were chromatographed on thin layer plates (Fig. 3). Practically the total amount of radioactivity (747 ±80 dpm) of the applied fraction could be detected on the ara-C

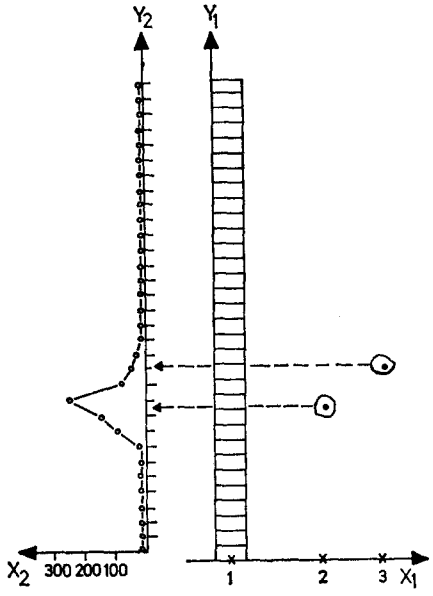

Fig. 3. Chromatographic separation of ara-C and dCyd by thin layer chromatography on cellulose. Separation conditions as described under methods.

x_1-axis: line of sample application to chromatogram, 1 : 50 µl pyrimidine-nucleotide fraction from Sephadex G 75 separation, 2 : 5 µg of ara-C in 50 µl of water, 3 : 5 µg of dCyd in 50 µl of water; y_1-axis: direction of development of chromatogram. Trace 1 was cut into strips and eluted; the position of ara-C (2) and dCyd (3) is indicated for comparison. x_2-axis: radioactivity in dpm; y_2-axis: number of strip measured from trace 1. Maximum radioactivity in the y-direction coincides with the ara-C position.

spot while only 50 dpm ±10 were eluted from the dCyd spot. This fact indicates that at least ca. 90 % of the radioactivity incorporated into DNA is due to ara-C.

The pyrimidine-nucleotide fraction (from Sephadex G-25) was also pooled and hydrolyzed again with DNase I, phosphodiesterase and alkaline phosphatase (the incubation time in this

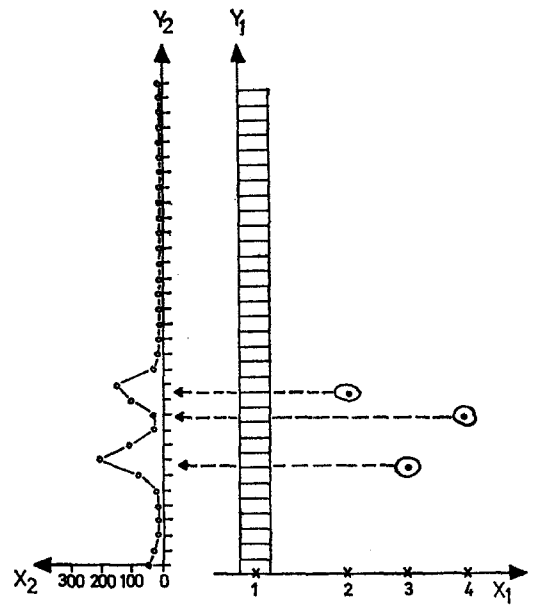

Fig. 4. Separation of Cyt, D-arabinose and ara-C by thin layer chromatography on cellulose.

x_1-axis: line of sample application; 1: KOH-hydrolysate of ara-C-^{3}H-DNA, 2: Cyt, 3: D-arabinose and 4: ara-C. y_1-axis: direction of development of chromatogram; trace 1 was cut into strips and eluted. The position of the other substances is indicated; x_2-axis: radioactivity in dpm; y_2-axis: number of strips. The two maxima of radioactivity in the y-direction coincide with the positions of cytosine and arabinose.

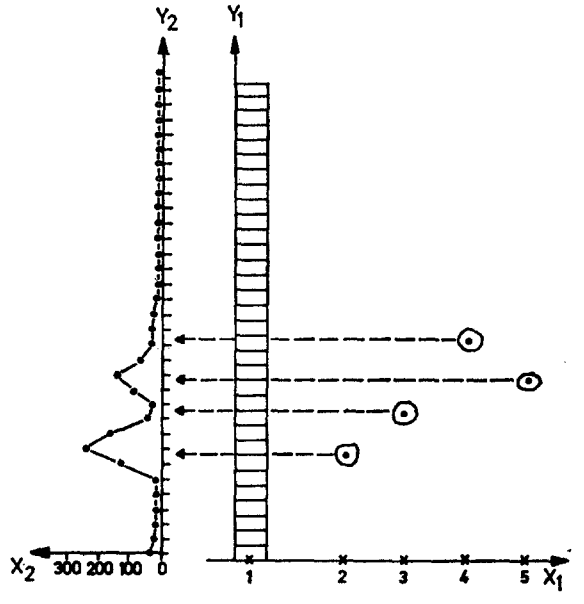

Fig. 5. Separation of ara-C-DNA hydrolysate.

x_1-axis: line of sample application to chromatogram, 1: KOH-hydrolysate of ara-C-^{3}H-DNA, 2: arabinose, 3: ribose, 4: deoxyribose, 5: cytosine. y_1-axis: direction of development. Trace 1 was cut into strips and eluted; x_2-axis: radioactivity in dpm; y_2-axis: number of strip measured from trace 1. The two maxima of radioactivity in the y-direction coincide with the positions of cytosine and arabinose, as in the test chromatogram Fig. 4.

case was 16 hr). After concentration *in vacuo* the hydrolyzed DNA products were fractionated on Sephadex G-25. Radioactivity could only be found in the nucleoside fractions (K_d = 1·1–1·3). As described above this radioactivity is clearly due to ara-C.

Other aliquots of enzymically digested ara-C-DNA fragments were hydrolyzed with KOH. By this method an almost complete cleavage of nucleosides into Cyt and D-arabinose is caused (Fig. 4). This KOH hydrolyzed sample was analyzed by thin layer chromatography. In the solvent used Cyt and D-arabinose can be separated from D-ribose and deoxy-D-ribose. As shown in Fig. 5 almost the total amount of radioactivity could be recovered on the arabinose- and Cyt-spot.

DISCUSSION

The data presented in this paper show, that the suggested incorporation of ara-C into DNA [7, 8, 1] can be demonstrated thoroughly. The argument against this incorporation raised by Cohen [10] could be eliminated by the evidence that in the nucleoside- as well as in the nucleotide fraction arabinose is associated with labeled cytosine. We found that at least 87% of the radioactivity incorporated into the DNA are due to ara-C. Only 6% can be attributed to dCyd. Therefore we suggest that from all the ara-C molecules incorporated into DNA, less than 6% are metabolically altered.

We found that per $1{\cdot}98 \times 10^3$ moles of DNA nucleotide 1 mole of ara-C is incorporated into DNA. This ratio is much higher than that ($2{\cdot}7 \times 10^4$: 1) found by Silagi [1].

In contrast to the results of Silagi [1] and Chu *et al.* [20] we could not detect any appreciable ara-C incorporation into RNA. This divergence, in our opinion, is due to the different methods used for separation of RNA from DNA. The isolation method applied by us seems to be more selective for large RNA molecules of possibly slow metabolism.

As ara-C incorporation into DNA is a fact the question arises if the inhibition of DNA synthesis in mammalian cells [1, 2] and viruses [3] is caused by this incorporation. It may be that by incorporation of ara-C into DNA a change in the structure of the DNA molecule results due to the more space filling trans C 2′ OH groups. By this the proper function of the DNA polymerase may be affected. On the other hand it can be expected that at least some inhibition observed in isolated DNA polymerizing enzyme systems [21, 22] via the triphosphate stage occurs also in intact biological systems. This will be discussed in another paper [23].

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Protection Against Graffi's Leukemia in Mice Treated with Leukemic Cells Photo-inactivated by Psoralen

LUIGI MUSAJO and FRANCAROSA BACCICHETTI

Istituto di Chimica Farmaceutica dell' Università, Centro di Studio per la Chimica del Farmaco e dei Prodotti biologicamente attivi del Consiglio Nazionale delle Ricerche, Padova, Italy

WE PREVIOUSLY reported that Ehrlich ascites tumor cells lose the capacity to induce tumours following irradiation at 365 nm in the presence of skin-photosensitizing furocoumarins (psoralens) [1]. The loss of the capacity to induce tumours is connected with the linkage of furocoumarins to nucleic acids. In fact, DNA extracted from ascites tumour cells previously irradiated in the presence of psoralen – ^3H, after hydrolysis releases a substance which is identical to the fluorescent photoadduct already obtained from psoralen and thymine [2]. The linkage of psoralen to DNA occurs only following irradiation. This is in agreement with our observation that furocoumarins lead to a C_4 – cycloaddition to the pyrimidine bases of the nucleic acids [3–5].

Recently we have also ascertained that in these cells irradiated as above nucleic acid synthesis, determined as incorporation of tritiated thymidine and uridine, is markedly depressed [7]. However, respiration, electron microscopy picture, and tests with Wrights' liquid and trypan blue occur as in untreated cells. These data suggested that these treated cells lose their dividing property but most probably do retain their antigenic structures. With that in mind, we succeded in demonstrating that such cells, photo-inactivated with psoralen or with 8-methylpsoralen, injected intraperitoneally at doses of 10^7 cells/mouse four times, give a significant protection towards 2×10^3 transplanted untreated tumour cells. Out of 219 animal controls, 168 died within 60 days, equivalent to 76·7 %; out of 271 treated animals 94 died in the same period, equivalent to 34·7 % [8].

We now report the results obtained extending these researches to a mouse leukemia originally induced by Graffi's virus in a $C_{57}BL$ mice, supplied from the Laboratory of Experimental Oncology of Padua University, at 92nd transplant generation in syngeneic animals.

Such a leukemia in our animals of the $C_{57}BL/6$ strain in dose of 10^6 cells/mouse subcutaneously injected gives an 80 % mortality; in dose of 10^7 a 100 %, with a mean survival time of 10 days.

The cellular suspension has been prepared taking the spleen of the animals in which the tumor was transplanted 10 days before and diluting with Hanks's solution in order to have 2×10^7 cells and 10 μg of psoralen per ml and irradiating for 30 min as previously described [1] (Philips HPW 125 lamps; 365 nm; irradiation intensity $1{\cdot}07 \times 10^{15}$ quanta/cm^2/sec.). Each animal to be protected received subcutaneously 1 ml of such a suspension. The photo-inactivated cells were administered 4 or 6 times at intervals of 7 days.

The challenge with leukemic cells, made subcutaneously 7 days after the last protective injection, was 10^6–10^7 leukemic cells. The results with relative controls are reported in Table 1.

Part of the treated animals, 60 days after the challenge, have been infected again; 10 animals with 10^6 and 50 with 10^7 cells; a survival of 100 %, observed for 60 days, has been obtained.

We may conclude that the animals treated as such are resistant to a challenge both with 10^6

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Table 1. *Protection observed in mice* $C_{57}BL$ *injected with leukemic cells irradiated at* 365 *nm in the presence of psoralen.*

No. mice	No. injection of 2×10^7 irradiated cells in the presence of psoralen	Challenge (No. cells)	Mortality No. dead mice / No. total mice
10	0	10^6	8/10
10	4	10^6	0/10
33	0	10^7	33/33
63	6	10^7	0/63

and 10^7 infecting cells. This resistance remains unchanged even after 60 days from the first infection. We can deduct that the leukemia cells of Graffi's virus photoinactivated with psoralen produce most probably a high level of lasting immunity in the mouse.

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Chromosomal Characteristics of Secondary Human Brain Tumours

JOACHIM MARK

Institute of Pathology, Department of Neurosurgery, and Institute of Genetics, University of Lund, S 221 85 Lund, Sweden

Abstract—*The chromosomes in 20 secondary neoplasms in the CNS were studied in fixations taken directly from the tumours. Three quarters of the metastases had a triploid mode, and three quarters of these were in the hypotriploid zone. Near-diploid stemlines were next in frequency; a hypodiploid mode was most common, and 2 such tumours had as low stemline number as 34 and 36, respectively. Tetraploid stemlines were not seen, but 1 metastasis had a pentaploid mode. In ordinary chromosome groups the deviations particularly involved groups C, D and G. Markers, and often a very high number, were ou nd in a ll but one stemline. A statistical analysis of the chromosomal representation demonstrated a correlation between the extent of the decrease of the acrocentrics and the frequency of markers. A study of the relative size of four different marker types indicated that these were formed by non-random mechanisms.*

In comparison with findings in neoplastic effusions the brain metastases showed more extreme numerical and structural features, possibly a property of neoplastic elements with a capacity to metastasize to the CNS.

INTRODUCTION

A COMPREHENSIVE study of the chromosomes in primary human tumours of the nervous system has been carried out [1–7] during the past 3 years. The last group to be considered consists of secondary neoplasms. The findings in this material are of interest when compared with observations in cancerous effusions [8]. The latter results represent the main source of our present knowledge about the chromosomal characteristics in metastatic tumours. Little attention has been paid to the fact that neoplastic effusions represent a fairly specific type of tumour spread, and that the metastases in solid forms and at other sites might show different chromosomal patterns.

MATERIAL AND METHODS

Table 1 shows the clinico-pathological characteristics of the material. It consisted of 20 metastases derived from 17 patients. Two different metastases from the same patient were studied in 3 cases, 1a, b, 8a, b and 13a, b, respectively.

The chromosomes were analysed in fixations taken directly from the tumour. The method was described previously [6, 7]. Fifty metaphases were counted in all metastases except No. 13a (25 cells) and No. 4 (100 cells). Karyotype analyses were made by photography and comprised 12 to 25 cells in all tumours except 13a (8 cells).

As in previous reports [1–7] S was used as a symbol for the stemline. Different morphological types of markers were designated with symbols in accordance with the nomenclature proposed by Levan *et al.* [9].

RESULTS

Numerical findings

Figures 1 and 2 show the distribution of chromosome counts in each metastasis. The predominating cell group, the modal population, was in the diploid region in 4 cases, in the triploid region in 15 cases and in the pentaploid zone in 1 case. None of the metastases had a tetraploid mode. The mode was usually flat

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Table 1. *Clinico-pathological features of the material.*

Case No.	Patient: Sex	Patient: Age (yrs)	Patient: Duration of symptoms prior to operation, (months)	Patient: Survival period, (months)	Metastasis: Site in the CNS	Metastasis: Histological type	Site of the primary tumour
1a	F	57	3	7	Temporal reg.	Poorly diff. adenoca.	Lung
b	F	57	3	7	Temporal reg.	Poorly diff. adenoca.	Lung
2	M	68	4	8	Parietal reg.	Oat cell ca.	Lung
3	F	60	2	8	Temporal reg.	Poorly diff. adenoca.	Lung
4	M	63	< 1	2	Parietal reg.	Anaplastic ca.	Lung
5	M	59	6	8	Cerebellum	Poorly diff. adenoca.	Lung
6	M	64	2	2	Occipital reg.	Anaplastic ca.	Lung
7	M	73	4	< 1	Parietal reg.	Anaplastic ca.	Lung
8a	M	60	4	> 20	Parietal reg.	Poorly diff. adenoca.	Lung
b	M	61	1	> 12	Parietal reg.	Poorly diff. adenoca.	Lung
9	M	76	2	5	Parietal reg.	Anaplastic ca.	Lung
10	F	39	1	> 8	Occipital reg.	Poorly diff. adenoca.	Kidney
11	M	63	1	5	Parietal reg.	Poorly diff. adenoca.	Kidney
12	M	68	3	3	Temporal reg.	Poorly diff. adenoca.	Pancreas
13a	F	50	1	3	Occipital reg.	Poorly diff. adenoca	Breast
b	F	50	2	< 1	Cerebellum	Poorly diff. adenoca.	Breast
14	M	69	1	2	Parietal reg.	Poorly diff. adenoca.	Colon
15	M	67	< 1	> 9	Parietal reg.	Melanoma	Chest
16	M	64	3	< 1	Occipital reg.	Melanoma	?
17	F	64	3	< 1	Frontal reg.	Melanoma	Leg

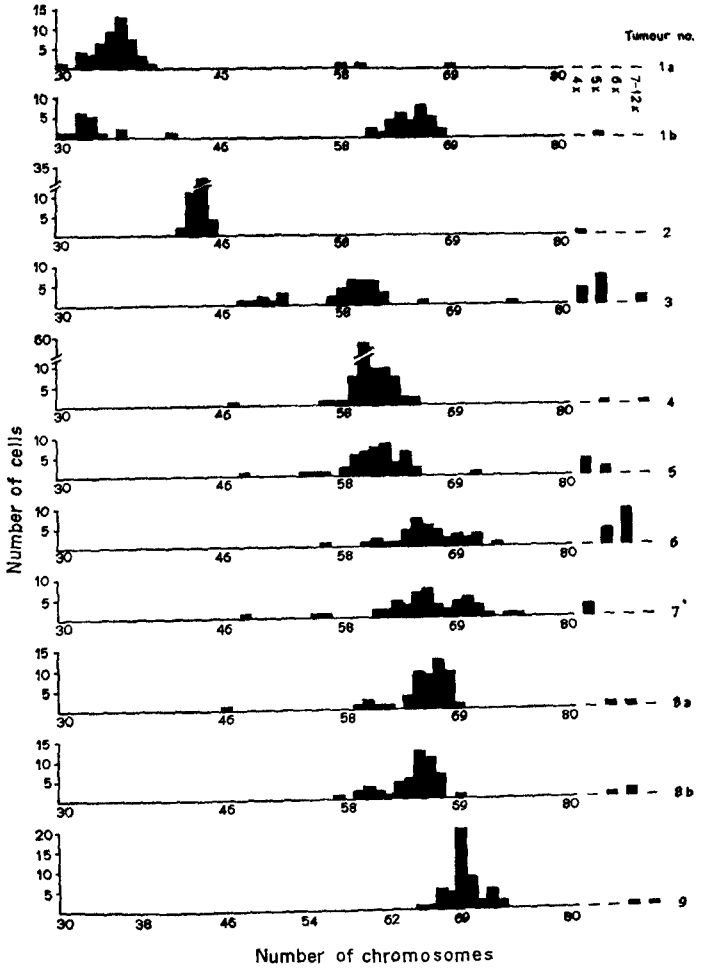

Fig. 1. *Histograms showing the chromosome counts in metastases Nos.* 1–9.

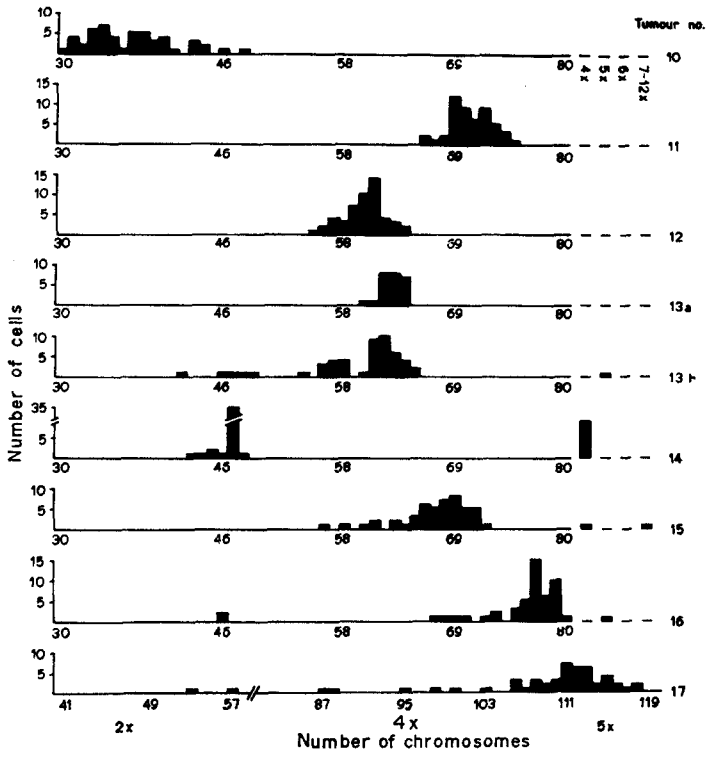

Fig. 2. *Histograms showing the chromosome counts in metastases Nos.* 10–17.

and often ill defined. The spread in the modal region was considerable, and in most cases a range of 10 to 15 chromosome numbers was involved. Several triploid cases showed a considerable fraction of high-polyploid cells, usually having chromosome numbers in the 2S-zone.

In order to achieve a picture of the total distribution of chromosome counts in the material, the counts in the 2 metastases Nos. 13a and 4 were readjusted to a sum of 50, as found in the other 18 tumours. The counts were subsequently pooled, and the distribution is shown in Fig. 3. Almost 65 % of all counts were in the triploid region; the hypotriploid counts were nearly 4 times as common as the hypertriploid ones, and the exact triploid counts constituted less than 10 % of the total counts in the triploid zone. A similar trend was also seen in the diploid region containing about 25 % of the total counts.

The pattern of normal chromosomes

Table 2 shows the karyotypic characteristics of the modal cells in each of the 20 metastases. The findings are recorded in relation to the normal, diploid complement in all cases except for tumour No. 17 which is listed in relation to a doubled normal female cell. There was often considerable variability among the analysed cells with the S-number(s), and in many cases it was impossible to find even two cells with an identical karyotype. This situation is accounted for in Table 2, which shows the

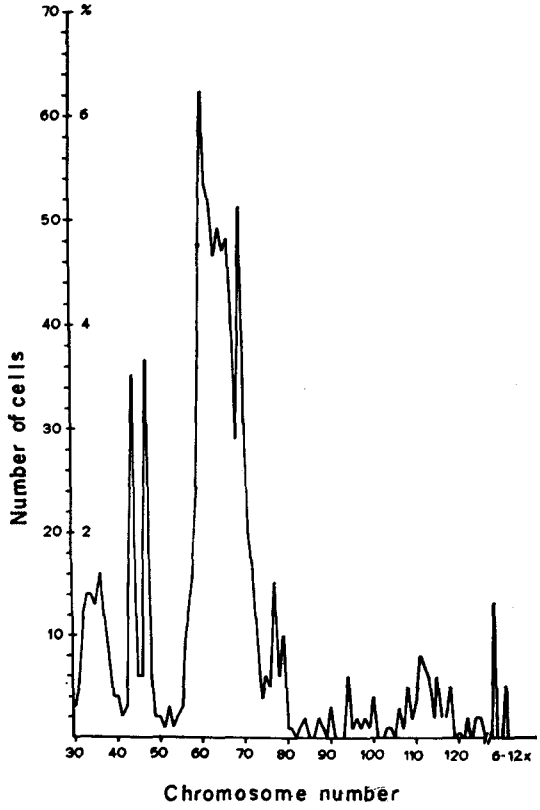

Fig. 3. *The distribution of chromosome counts in the entire material of brain metastases.*

Table 2. Karyotypic features of the modal cells in the brain metastases.

Case No.	Chromosome groups											Modal number(s)	% S-cells
	A1	A2	A3	B	C	D	E16	E17–18	F	G	Markers		
1a	−1	—	−0–1	—	−3–4	−2–3	−1	−2	−2–3	−2–3	+4–6	36	—
b	—	—	+1–2	+3–4	+4–6	+2	—	—	—	−2	+10–11	66	—
2	—	—	—	−1	+2	−1	—	—	−1	−1	—	44	44·8
3	—	+2	+3–4	+1–2	+1–4	−4–5	−1	−1	−2	—	+11–15	59–61	—
4	+0–1	—	+4–5	−2–3	+5–6	−0–2	—	—	+2	−4	+8–10	60	7·7
5	—	+1	+2	—	+6–8	−3	—	—	—	−0–2	+9–11	62	—
6	+1	—	—	−1	+6–9	+4–5	+2	+0–2	+4	−1–2	+2–4	65	—
7	+2	+2	+2	−1	+7–9	−1–2	+2	+2–3	—	−3	+7	66	7·0
8a	+1	—	+2	+1	+9–10	−1	+2	+2	+1	−1	+4–5	67	6·8
b	+1	−1	+2	+1	+8–9	—	+2	+2	+0–1	−0–1	+4	65–66	6·3
9	—	—	+1	+1	+19	−4	—	—	+2	+2	+2	69	20·0
10	−0–1	—	—	−1	−8–10	−0–2	−0–1	−1	—	−0–1	+1–2	34	—
11	—	+1–2	—	+1	+6–9	—	+1–2	+1–3	+2	−0–2	+7–8	69	—
12	—	—	—	−1	+6	—	+1	+2	+1	−1	+7	61	16·0
13a	+1	+2	+4	+2	+2–3	−1	—	+0–1	—	−1	+7	62–64	6·2
b	—	+2	+4	+1	+4–5	−1	−1	−1	—	—	+7	61–62	7·0
14	—	—	—	—	+1	—	—	—	—	−1	+1	47	64·2
15	—	+1	+0–1	+1	+3–9	+2–4	+1–2	+1–2	+2	+0–1	+4–7	68–69	—
16	−1	+1	+1	+4	+8	+5	+2	+4	+6	—	+1	77	15·0
17	−0–2	—	—	—	+10–14	−1–2	—	—	—	+3–4	+5–7	111–113	—

karyotypic features relevant to the majority of the modal cells, by indicating a more or less restricted range for the gains or the losses, in one or several chromosome groups.

A direct measure of the degree of karyological uniformity of the modal cells is the frequency of S-cells in the tumour cell population (last column in Table 2). This frequency is easily calculated from the results of the counts and the karyotype analyses. No frequency for the S-cells is given in nine cases, since in these tumours all of the analysed cells with the modal number(s) had a different karyotype. About half of the remaining eleven cases were characterized by such a low frequency of S-cells as 6 to 8 %. Tumour No. 4 belonged to this group, in spite of its prominent mode. The reason for this was that the modal elements consisted of many small subgroups, each with its own karyotype, and also many single cells with a deviating marker set. Only two tumours, Nos. 2 and 14, showed a high frequency of S-cells (about 45 and 64 %, respectively). Both neoplasms had their S in the diploid region, and both of them had an S-number close to the normal diploid one.

In the ordinary chromosome groups the pattern is complex but several trends can be discerned. Thus, group C was involved in all metastases, and the deviations consisted of gains in all but two cases, namely those with an extreme hypodiploid mode, Nos. 1 and 10, respectively. The deviations in group C, gains or losses, tended to increase with increasing deviation from the normal, diploid chromosome number. Next to group C, the small acrocentrics were most frequently involved (85 %). Losses were the rule in group G, the metastases Nos. 9, 15 and 17 being exceptions. A similar pattern prevailed in group D, which was third in frequency of involvement together with group B, both groups being involved in 80 % of the stemlines. In group B there was no consistency in the pattern of deviations, nor was this seen in group E17–18, F and E16, which were fourth in frequency of engagement together with A3. In the group A3, however, 12 of the 13 affected stemlines showed gains. A similar picture was also seen in group A2 which was least frequently involved together with A1 (45 and 50 %, respectively).

The pattern of marker chromosomes

Markers occurred in the stemlines of all tumours except for No. 2. Among these 19 metastases, 17 had more than one marker, and 9 of those with multiple markers had as many as 7 or more markers in their S-cells. In the latter group of 9 tumours all except one had its mode in the 3x-region, indicating a correlation between extensive structural rearrangements and near-triploid S-numbers.

The marker types usually found in the modal cells are illustrated in Fig. 4. These condensed karyotypes demonstrate the size and morphological characteristics of the markers in relation to a reference set of ordinary chromosomes picked out from the complete karyotypes.

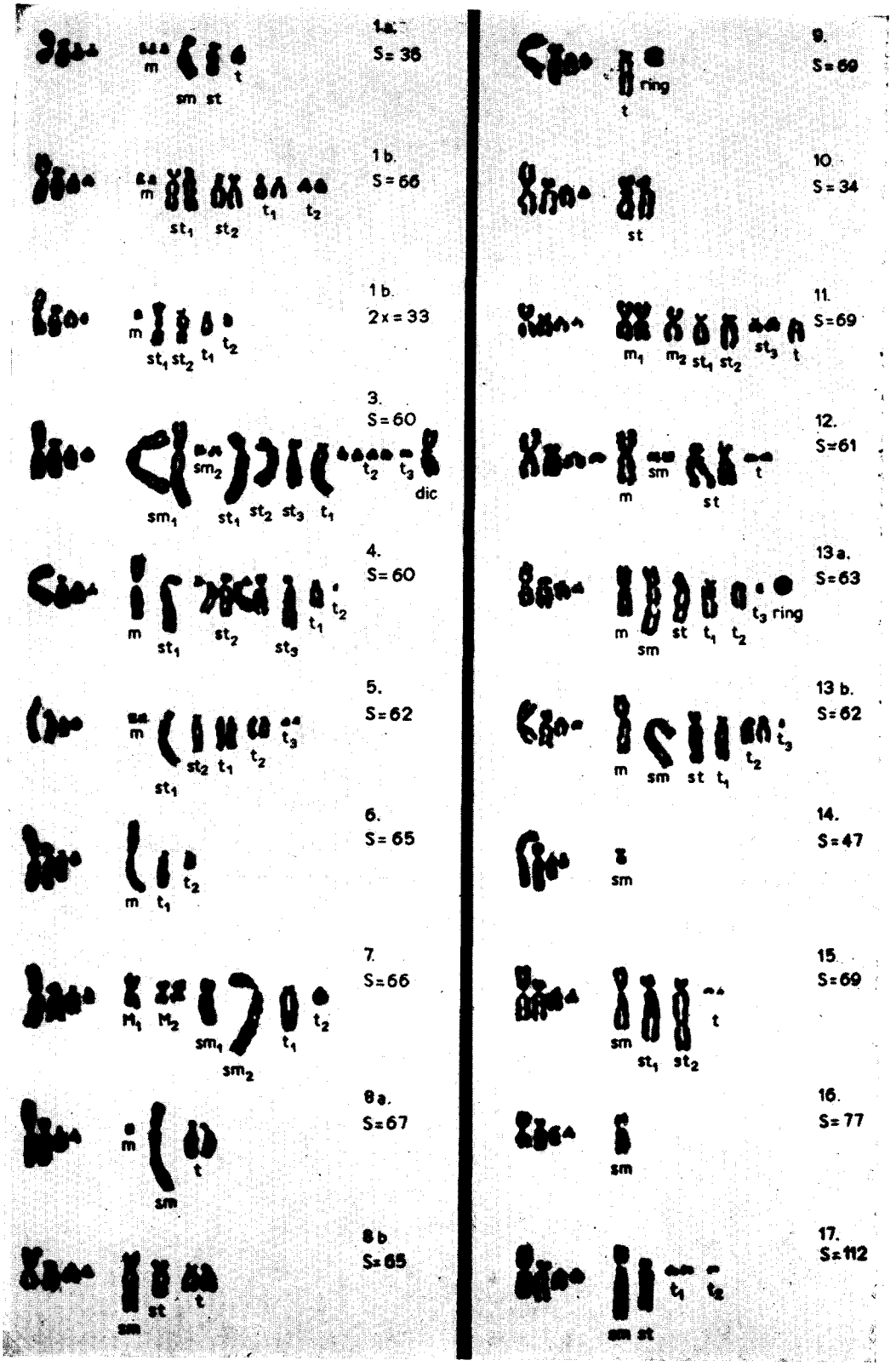

Fig. 4. *Condensed karyotypes of a typical modal cell (and one variant cell in tumour No.* 1*b*) *in all metastases with markers in their stemline (markers were absent only in tumour No.* 2). *In all cases the marker set is shown in relation to four normal chromosomes, represented by one A*1 *and one each of the biggest B, D and G chromosomes.*

(to face p. 402)

This reference set always consists of the same chromosomes, namely one A1 and one each of the biggest B, D and G chromosomes; the differences between nearly all markers, and the ordinary chromosomes they might be confused with, were shown by this choice of reference chromosomes.

Three marker types were rare, namely dicentrics, M-markers and ring chromosomes. A dicentric with varying size and morphology was incorporated in the extensive marker set of tumour No. 3. Three M-markers were found in the marker set of metastasis No. 7. One ring chromosome characterized the modal cells in metastases Nos. 9 and 13a. In each tumour the ring chromosome had a similar size in all analysed cells, and accordingly this marker in both cases seemed to be a stable component of the karyotype.

Except for the rare marker types, there were altogether 106 markers in the stemlines of the 20 metastases. These markers belonged to 4 different types, t, st, sm and m, respectively. As many as 43 and 30 were of the t- and st-types, respectively, whereas the number of sm- and m-types amounted to only 17 and 16, respectively. The markers could always be distinguished by their abnormal size and/or arm-ratio; however, the occurrence of one or several constant secondary constrictions in some markers was often an additional helpful hallmark (Fig. 4, tumours Nos. 1a, b, 10, 13a, b, 15 and others).

The size distribution for the t-, st-, sm- and m-markers is shown in the histograms of Fig. 5. The size of each individual marker has been calculated as a percentage of the average size of the A1 chromosomes in the analysed cell, and the figures used represent the mean values after measurements of each marker type and the corresponding A1 chromosome in 3 to 5 modal cells. The histograms show that the pattern for the size distribution was different for the four marker types. It is also obvious that certain size classes were predominant for each marker type.

It can be seen that the modal features in the marker groups t and st are true characteristics. This also seemed probable for the more distinctive trends in groups sm and m, in spite of their often less prominent peaks, and in spite of the greater influence of normal chromosomes on the pattern. The cause of the modal features is explained by (1) properties of chromosome organization, (2) the mechanisms for the structural rearrangements, and (3) the particular chromosomes involved in the rearrangements.

As regards (1), the results indicate that a distal location of the centromere leads to instability in big chromosomes, hence the absence of t-markers in the size classes above 100 % and the few cases in the same size range among st-markers. (2) The types of structural rearrangements to be expected most frequently are the least complicated ones, namely deletions

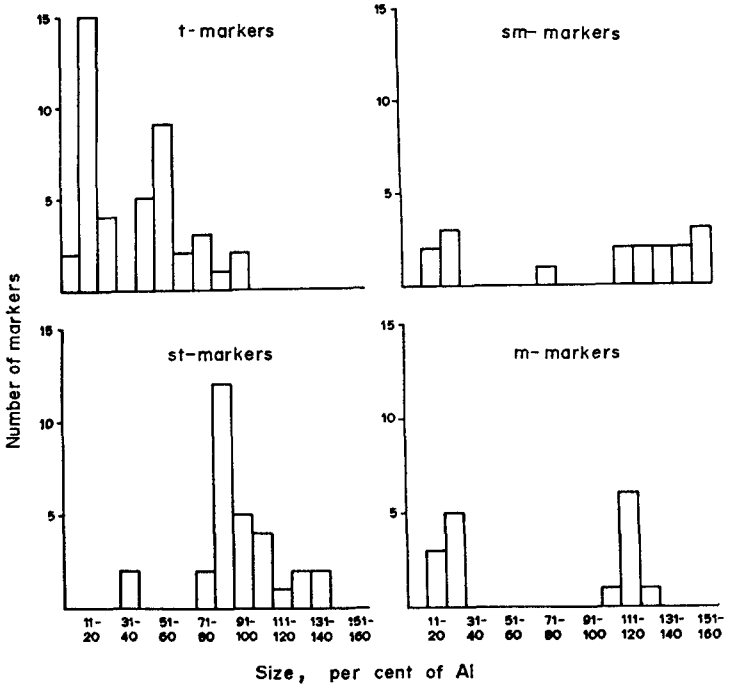

Fig. 5. Histograms showing the size distribution of all markers of the types t, st, sm and m in the brain metastases; the size is expressed as a percentage of the average size of the chromosome No. A1 (= 100%).

or deficiences. Such aberrations, affecting various normal chromosomes, could account for the formation of all small sm- and m-markers, all st-markers up to a size of about 95% (of the normal A1), and for the appearance of all t-markers up to a size of about 70%. The upper limit for st- and t-markers is a result of the definition for the arm-ratio for markers of the st-t-types and the relative length of the longest chromosome arms of the normal chromosomes (A2, B4, A1 and B5). If the t-markers with a size just below 70% had usually been formed by deletions, as also the st-markers with a size just below 95%, there had to be an obvious corresponding loss of A and/or B chromosomes. Such losses were neither a consistent nor a very common feature in the stemlines with such markers. Thus, translocations or more complicated rearrangements must be involved in the formation of the bigger t-st-markers. A simple translocation of one piece of a chromosome to another could not account for the formation of the big sm- and m-markers, unless the A and/or B chromosomes were involved both as a donor and a recipient. The reasons are the same as those discussed above. The karyotypic findings in stemlines with big markers of m-sm-types only support such a possibility in a few instances. Accordingly, more complicated mechanisms must be considered to explain the formation of most big m-sm-markers. Only one possible mechanism deserves mention, namely a secondary remodelling of markers already formed. This is because newly formed chromosomes are likely to be involved in further structural rearrangements.

(3) The chromosome(s) involved in the formation of a marker was usually impossible to decipher due to the common engagement of many ordinary chromosome groups. Metastasis No. 14 was the only exception. In this case it seemed probable that the m-marker was derived from a missing G chromosome with a translocation on the short arm. The origin of the translocated part, however, was uncertain.

The chromosomal representation

To obtain an idea of the character of the chromosomal representation in the metastases, a statistical analysis was carried out according to principles outlined earlier by Levan [10]. In each tumour, all karyotyped cells in the S-region (8 to 21 cells) were used to calculate the mean differences between observed and expected values in the 11 chromosome groups of Table 3.

The most striking features are seen in groups D, G, C and markers. In both groups of acrocentrics there was a negative value for all tumour types (less chromosomes than expected). As a rule the negative value was significant, that is higher than 0·5. There was no difference between the small and big acrocentrics with regard to the extent of the deviations. The positive value for the group markers was a result of the method used but the very high values in most groups of tumours deserve attention. In the other chromosome groups there was a tendency towards losses especially in groups A1 and B and less so in groups E17–18 and F, and there was a trend towards proportional gains in group C and less so in group A3. The pattern in the other chromosome groups was inconsistent. The findings led to an analysis of the relation between the losses among the acrocentrics and the gains of markers and C chromosomes. This relation is shown diagrammatically in Fig. 6. The tumours were arranged according to an increasing number of markers (calculated as in Table 3), and the values for

Table 3. Mean differences between observed and expected values in the brain metastases.

Site of the primary tumour	Histological type	No. of tumours	Chromosome groups										
			A1	A2	A3	B	C	D	E16	E17–18	F	G	Markers
Lung	Oat cell ca.	1	+0·1	+0·1	+0·1	−0·8	+2·1	−0·7	+0·1	+0·2	−0·8	−0·7	+0·3
	Anaplastic ca.	4	+0·0	−0·3	+0·9	−2·4	+3·5	−3·2	+0·2	−0·6	+0·3	−3·6	+5·3
	Adenoca.	6	−0·6	−0·2	+1·2	+0·2	+0·1	−3·1	−0·5	−0·9	−1·1	−2·5	+7·6
	All types	11	−0·3	−0·2	+1·0	−0·8	+1·5	−2·9	−0·2	−0·7	−0·6	−2·7	+6·1
Kidney	Adenoca	2	−0·6	+0·3	−0·2	−0·8	−1·0	−1·0	+0·2	−0·3	+0·1	−1·6	+4·9
Pancreas	Adenoca.	1	−0·6	−0·6	−0·6	−2·3	+0·6	−1·9	+0·3	+0·7	−0·3	−2·6	+7·2
Breast	Adenoca.	2	−0·1	+1·5	+3·0	+0·1	−3·0	−3·0	−0·9	−1·2	−1·3	−1·8	+6·5
Colon	Adenoca.	1	−0·0	−0·0	−0·0	−0·1	+0·7	−0·1	−0·0	−0·1	−0·1	−1·1	+1·0
Skin	Melanoma	3	−1·6	−0·4	−0·5	−0·4	+0·2	−1·0	+0·2	+0·0	+0·7	−0·8	+3·6
Mean of total material		20	−0·5	−0·0	+0·7	−0·7	+0·5	−2·2	−0·1	−0·5	−0·4	−2·2	+5·4

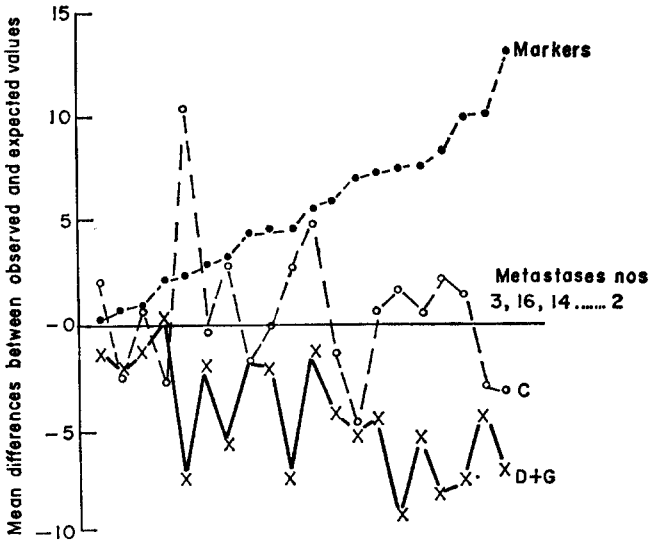

Fig 6. *Diagram showing the relation between the chromosomal representation in groups markers* (-·-·-·-·-), *C* (— —○— —) *and D+G* (—— ×——) *in the* 20 *brain metastases, the latter being arranged in the order of an increasing number of markers.*

groups G and D have been added for each tumour. It is obvious that there is an increasing proportional loss of acrocentrics with a rise in the average number of markers. This undoubtedly indicates that the acrocentrics play an important role in the formation of markers. No relation, however, could be found between the pattern of group C and that of the acrocentrics and markers. This is specifically mentioned, since previous similar analyses of secondary tumours have shown fairly consistent high positive values in group C [5, 8].

The number of metastases in each histological group of Table 3 is varying and usually very small, which makes all comparisons difficult. As far as the present data are concerned, however, there were no obvious differences between the various groups of secondary tumours.

DISCUSSION

The chromosomes of 3 secondary human tumours in the brain have been studied earlier. A metastasis of a lung carcinoma had a hypodiploid mode, S = 34, but no karyotypes were given [11]. The other 2 metastases were anaplastic carcinomas; in both cases the site of the primary tumour was unknown [12]. One of these had a hypotriploid stemline, S = ±61; no markers were seen in the two cells karyotyped. The other tumour had a psuedodiploid stemline with 1 to 2 markers. Together with the present material these scanty observations can be used for a comparison with the findings in other human metastatic lesions. The overwhelming majority of these lesions consists of cancerous effusions.

Table 4. *The distribution of modal numbers in cancerous effusions* (129 *cases*) *and in brain metastases* (23 *cases*).

Level of ploidy	Modal range	Cancerous effusions	Brain metastases
	33–45	17·0	17·3
Diploidy	46	14·0	4·4
	47–57	17·0	4·4
		48·0	26·1
	58–68	15·5	56·5
Triploidy	69	0·8	8·7
	70–80	23·3	4·4
		39·6	69·6
	81–91	6·2	0
Tetrahexaploidy	92	0	0
	93–133	6·2	4·4
		12·4	4·4

Table 4 is a comparison between the distribution of modal numbers in the 23 secondary brain tumours and that of 129 cancerous effusions. The values in the latter material were recently published by Sandberg *et al.* [8]. The findings in secondary brain tumours differed from those in cancerous effusions in several respects. In the former material, about 70% of the neoplasms had their stemline in the

triploid region, whereas in the effusions this frequency was only about 40 %. In the 3x-group the hypotriploid stemlines predominated in the brain metastases, whereas in the effusions the hypertriploids were more common than the hypotriploids. In almost half of the effusions the mode was in the diploid region, and within this region the cases with an exact diploid mode were nearly as common as the hypo- and hyperdiploids. Only about one quarter of the brain metastases had their stemline in the diploid region, and the hypodiploids were more common than the other 2 groups. In the ploidy groups above the triploid one, the total frequency was much lower in secondary brain tumours, which was mainly due to the absence of tetraploid stemlines. In both effusions and brain metastases, however, the mode was usually flat, and the frequency of cells with the S-number(s) was low, often very low.

Differences are also seen between cancerous effusions and secondary brain tumours, as regards the karyotypic findings. Thus, Sandberg *et al.* [8], comparing their findings in effusions and solid tumours, reported a frequency of stemlines with markers of about 75 % in effusions versus about 50 % in solid tumours. All but 2 of the secondary brain tumours had markers in their stemline, a frequency of more than 90 %. In addition, a large number of markers in the modal cells was unusual in solid tumours compared with effusions. The average frequency of markers in 18 tabulated effusions of Sandberg *et al.* would be about 2·5. In secondary brain tumours the corresponding average value for the frequency of markers was about twice that in the effusions. The findings in the brain metastases also indicated a correlation between the development of triploid stemlines and a very high frequency of markers in the modal cells.

The karyotypic findings in ordinary chromosome groups in effusions are described later on [13] as inconsistent, no particular group being involved more often than another. Levan [10] and van Steenis [14], using mainly effusions for their statistical analyses, arrived at a different conclusion; their results agreed and showed a significant proportional loss of the small and big acrocentrics and also significant gains in group C, in addition to the group markers. The findings in the present study agree with the results of the latter investigators, except for a much higher positive value for the group markers. This value is, in fact, the highest one so far recorded in any material subjected to this type of statistical analysis. In the brain metastases the statistical findings also indicated a correlation between the number of markers and the extent of the total loss of acrocentrics (D + G chromosomes).

Summarizing the above discussion, metastatic brain tumours compared with cancerous effusions, showed a much higher frequency of triploid stemlines, a higher frequency of stemlines with markers and also a higher average number of markers in the modal cells. Finally, in ordinary chromosome groups the brain metastases showed a similar proportional loss of the small and big acrocentrics, as seen in the effusions, but the tendency towards gains in group C was much less pronounced.

The reasons for the chromosomal differences between secondary brain tumours and cancerous effusions are difficult to evaluate. The possible influence of the physical state of the tumour, effusion form versus solid form was mentioned in the introduction. A related question concerns the influence of differences between the tumour types included in each of the 2 materials. There is also the possibility, however, that the more extreme numerical and structural characteristics of the stemlines in the brain metastases are typical features of neoplastic elements of a certain tumour, capable of spread to and growth in the CNS.

The present study has also thrown light on another interesting question, namely the chromosomal picture in different metastases from the same primary tumour. The data, related to observations in 2 different metastases in 3 patients (Nos. 1, 8 and 13) demonstrated a similar picture, as to S-numbers and karyotypic findings, in 2 cases (Nos. 8 and 13). In the third case, No. 1, one metastasis had a hypodiploid stemline and the other one a hypotriploid mode; the stemline in the latter tumour was not a simple doubling-product of hypodiploid elements with characteristics as in the other metastasis. Thus, the relation between the evolutionary pathways in the two metastases could not be deduced. The occurrence of several marker types with identical characteristics in both tumours, however, revealed their common origin. The findings in the last case correspond well with the pronounced chromosomal pleomorphism seen in many primary tumours, and they illustrated the existence of different subclones with a similar invasive capacity within the primary tumours.

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Methotrexate Toxicity: Correlation with Duration of Administration, Plasma Levels, Dose and Excretion Pattern

J. H. GOLDIE*, L. A. PRICE† and K. R. HARRAP‡

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London SW3 6JB.

Abstract—*Thirty intravenous infusions of methotrexate in doses ranging from* 250 *mg/m² to* 12,000 *mg/m² for times varying from* 12 *to* 48 *hr were given to* 8 *patients with various malignant diseases. The plasma concentrations and excretion rates of methotrexate were measured. The toxicity of the drug proved to be related only to the time during which the infusion was given and not to the dose. In this series it was shown that if certain precautions were rigorously observed then (a) methotrexate toxicity could nearly always be avoided, (b) much larger doses than those usually employed could be given, and (c) methotrexate resistance could occasionally be overcome.*

INTRODUCTION

METHOTREXATE (M.T.X.) has been used clinically in various dose schedules in many different malignant diseases. Djerassi gave doses up to 1050 mg/m² in short infusions lasting up to 6 hr [1]. Bergsagel [2] gave doses of 75 mg/m² intravenously every 8 hr, and found that when the duration of exposure to MTX exceeded 48 hr serious haematological and gastrointestinal toxicity developed. Mitchell *et al.* [3] gave 240 mg/m² as a continuous infusion over 24 hr. Other workers have also given high doses of MTX, notably Schwarzenberg *et al.* [4]. Folinic acid (Leucovorin) has generally been used to encourage the recovery of rapidly proliferating normal cells and so reduce toxicity that would otherwise occur.

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*R. S. McLaughlin Travelling Fellow. Present position: Attending Physician, St Michael's Hospital, Toronto, and Associate, Department of Medicine, University of Toronto.

†Gordon Jacobs Research Fellow.

‡Head, Department of Applied Biochemistry.

In this study we have given much larger doses of MTX than those indicated above in an attempt (*a*) to define the limits of tolerance to the drug and (*b*) to overcome methotrexate resistance in human tumours. In order to assess these aims objectively, we felt that it was not enough simply to relate the dose given to the effect obtained. It is well known with many drugs that the same dose when administered to people of the same weight may produce marked differences in the plasma levels of the drug and hence in its therapeutic effects. There are several reasons for this. There may be genetically determined variations in the rate of breakdown of the drug, as with isoniazid [5], or other drugs given concurrently may influence the plasma level or the metabolism of the drug under study by displacing it from carrier protein as with sulphonamides and tolbutamide [6], or by inducing hepatic enzymes to break it down more rapidly as with barbiturates and anticoagulants [7]. Lastly, a patient with impaired renal function will retain the drug for a longer time. Thus, in the case of MTX, a statement of the dose in terms of mg/kg of body weight or

mg/m^2 of body surface area must be supplemented by serial measurements of the plasma levels and excretion rates during each infusion.

The rationale for achieving high plasma levels of MTX was based on the assumption that clinical resistance to the drug might develop by mechanisms similar to those occurring in certain experimental situations. Several such mechanisms have been described, e.g. reduction of cell-membrane permeability to the drug [8], increased synthesis of the target enzyme dihydrofolate reductase [9, 10] or detoxication of the drug [11]. We have previously reported [12] that an MTX-resistant line of L5178Y lymphoma cells in tissue culture could still be killed if the extra-cellular concentration of the drug was increased sufficiently. If similar processes contribute to resistance in human tumour cells, then increasing the extracellular concentration for a sufficient time might be expected to kill cells resistant to lower doses.

The reasons for supposing that massive doses of MTX could be given were based partly on the clinical work already indicated and partly on the experiments of Bruce *et al.* [13], who showed that under certain laboratory conditions many bone marrow cells were invulnerable to MTX, irrespective of dose, provided the drug was administered over a short period of time. This situation may apply to other proliferating systems such as the gastrointestinal tract. Consequently there should be no upper limit to the amount of MTX that could be given provided that the duration of administration was kept short. If so, it should be possible to give a sufficient amount of drug to kill those cells in human tumours which were resistant by virtue of the first two mechanisms described above.

In this paper we report the plasma concentrations that were obtained during infusions of various doses of MTX over various times. We

Table 1. *Diagnosis, previous therapy, number of infusions and response of each patient studied*

No.	Diagnosis	Previous therapy	No. of infusions	Response
1	Retropharyngeal liposarcoma	Radiotherapy, surgery, MTX, 5 FU, 6 MP	12	Yes
2	Fibrosarcoma mandible	DXR	4	Yes
3	Poorly differentiated lymphocytic lymphoma	Rubidomycin, 6 MP, cytosine	1	Yes
4	Malignant melanomatosis	Melphalan	4	No
5	Lymphosarcoma	MTX, cyclophosphamide, VCR, prednisolone, rubidomycin, cytosine	1	No
6	Fibrosarcoma	Radiotherapy, MTX	3	No
7	Melanomatosis	Cyclophosphamide, VCR, MTX, 5FU	1	No
8	A.L.L.	Prednisolone, 6 MP, VCR, Rubidomycin, L-asparaginase	4	Yes

Abbreviations: VCR: vincristine sulphate; 5 FU: 5 fluorouracil; 6 MP: 6 mercaptopurine; MTX: methotrexate; DXR: radiotherapy.

Key: 1. Smallest dose: 450 mg. Largest dose: 20,000 mg. Shortest infusion: 12 hr. Longest infusion: 48 hr.
2. Smallest dose: 5000 mg. Largest dose: 9000 mg. Shortest infusion: 24 hr. Longest infusion: 36 hr.
3. 2000 mg given over 20 hr.
4. Smallest dose: 750 mg. Largest dose: 5000 mg. Shortest infusion: 16 hr. Longest infusion: 24 hr.
5. 450 mg over 18 hr.
6. Smallest dose: 100 mg. Largest dose: 5000 mg. All infusions: 24 hr.
7. 8000 mg over 30 hr.
8. All doses 500 mg. Shortest infusion: 24 hr. Longest infusion: 36 hr.

also give the amounts excreted in the first 4 days and we attempt to relate the toxic and therapeutic effects to the total dose, the plasma concentration and the duration of the infusions.

PATIENTS AND METHODS

Eight patients were studied. All had tumours which were considered to be resistant to conventional therapy. Table 1 gives the diagnosis, previous treatment, and response to MTX of each patient. Assessment of partial response was objective, i.e. consisted of a reduction of at least 50% of some measurable aspect of the disease. Symptomatic improvements, however definite, were ignored. For administration, the MTX was dissolved in 1 or 2 l of N saline and the resulting solution was infused intravenously for times varying from 12 to 48 hr. The infusions were usually given every 14 days. The lowest dose given was 250 mg/m^2 (450 mg) and the highest 12,000 mg/m^2 (20,000 mg). In all patients, plasma and urinary MTX concentrations were measured at various times after the start of the infusion by the method of Bertino and Fischer [14]. Folinic acid (Calcium Leucovorin, Lederle) was given at the end of each infusion as a single intravenous injection equivalent to 20% of the dose of MTX infused, followed by 5 intramuscular injections of 15 mg each at 6-hourly intervals. For the 20,000 mg infusion, 600 mg of folinic acid was given at 24 hr (the end of the infusion) followed by two further i.v. injections of 600 mg each at 6-hourly intervals and then by 21 mg i.m. 6-hourly for 5 doses. The following investigations were carried out before and after each infusion: bone-marrow aspiration, creatinine clearance, peripheral blood count and film, blood uric acid, blood urea, serum bilirubin, alkaline phosphatase and alanine aminotransferase levels.

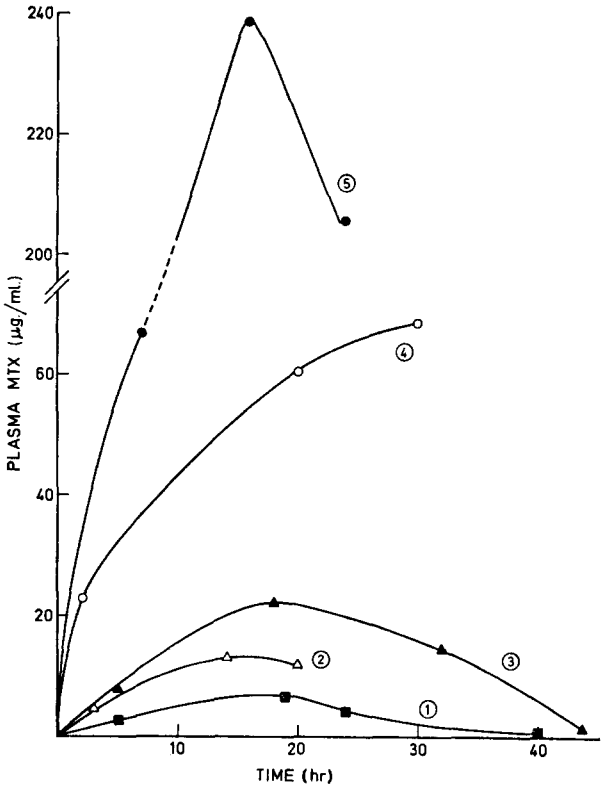

Fig. 1. Plasma MTX concentrations at various times after the start of infusion of different doses.
(1) = 750 *mg over* 24 *hr*, (2) = 2000 *mg over* 20 *hr*, (3) = 5000 *mg over* 36 *hr*, (4) = 8000 *mg over* 30 *hr*, (5) = 20,000 *mg over* 24 *hr*.
Note disproportionate rise in plasma concentration of MTX following infusion 5.

RESULTS

These are summarised in the form of tables and charts. Fig. 1 and Fig. 2, Table 2 and Table 3.

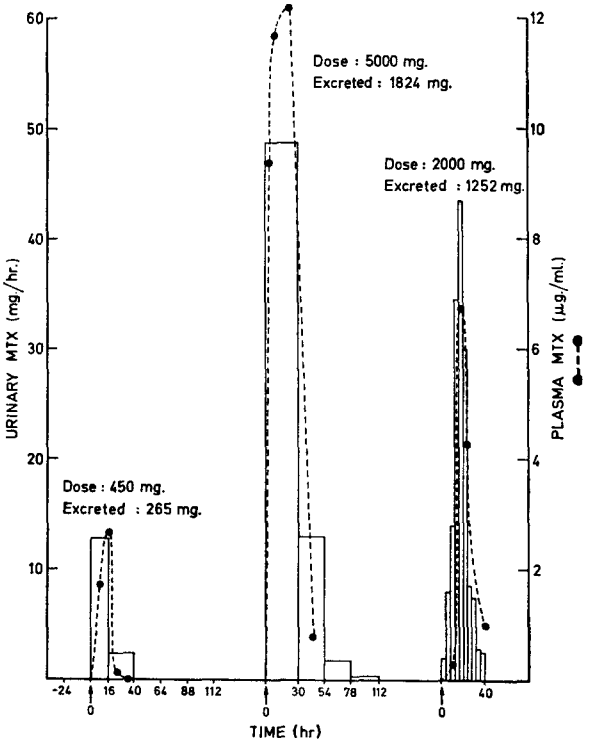

Fig. 2. Amounts of MTX excreted at 3 different doses. The corresponding plasma levels have been superimposed (interrupted lines).
Note (*a*) *the total amount excreted was always less than the amount infused.*
(*b*) *most of the excreted drug came out during the infusion.*
(*c*) *the plasma level fell sharply for* 6 *hr from the end of the infusion, and more slowly over the next* 24 *hr.*
All these patients had a normal creatinine clearance.

Table 2. Relationship between infusion time and haematological toxicity at different doses of MTX.

Dose MTX (mg)	Infusion time (hr)	Pretreatment total white count/μl	Pretreatment platelet count/cmm	Lowest post-treatment total white count	Lowest post-treatment platelet count
100	24	5,200	191,000	4,600	182,000
450	12	7,400	148,000	6,300	145,000
750	16	4,300	211,000	4,100	215,000
2,000	20	3,900	151,000	3,500	147,000
5,000	24	6,200	250,000	5,900	241,000
7,000	48	5,000	110,000	950	40,000
9,000	36	5,200	160,000	4,000	110,000
20,000	24	5,700	142,000	7,100	174,000

Note (1) Toxicity is independent of dose.
(2) Toxicity is proportional to the time of the infusion.

Table 3. Relationship between infusion time and gastrointestinal and hepatic toxicity at different doses of MTX. Gastrointestinal toxicity was assessed by oral ulceration.

Dose (mg)	Infusion time (hr)	Pretreatment serum alanine aminotransferase (normal = < 35)	Highest post-treatment serum alinine aminotransferase	Gastro-intestinal toxicity
100	24	7	9	None
450	12	11	6	None
750	16	10	11	None
2,000	20	5	8	None
5,000	24	13	11	None
7,000	48	29	73	Severe
9,000	36	14	40	Moderate
20,000	24	10	14	None

Note (1) Toxicity is independent of dose.
(2) Toxicity is proportional to the time of the infusion.

DISCUSSION

As shown in Tables 2 and 3 the toxicity of MTX was related only to the time of the infusion and never to the dose. This applied whichever index of toxicity was studied. Thus, hepatic, bone marrow and gut toxicity did not occur with infusions of 30 hr or less, but began to appear only in infusions lasting 36 hr or more, irrespective of the total dose administered. The longest infusion (48 hr) produced the greatest toxicity.

This relationship between toxicity and infusion time is well illustrated by patient No. 1 whose peripheral blood chart is shown in Fig. 3. There are two significant points about this chart. First, it is very flat, i.e. in spite of the large doses given, no catastrophic falls of Hb, white count or platelets occur. Secondly, more damage was done by 7000 mg over 48 hr (infusion 12) than by 20,000 mg over 24 hr (infusion 11), again emphasising that it is the duration of the infusion which is important and not the dose. Patient No. 1 had a retropharyngeal liposarcoma which was resistant to 5 mg doses of MTX given orally each day for 4 days in intermittent courses. Even so, he responded dramatically to 450 mg given over 16 hr. This meant that in this case at least resistance to MTX as usually given could be overcome by giving the drug by this different method. We feel, therefore, that the phrase "resistant to MTX" should not be used without specifying the way in which the drug is given.

Patients 5, 6 and 7, who were resistant to MTX given as 50 mg doses on two days a week, did not respond at all when the drug was infused. Responses were seen in patients 2, 3 and 8, none of whom had had MTX previously.

As seen in Fig. 2, between 37% and 62% of the infused drug was retained in the patient's tissues. We do not yet know the fate of this retained drug, but there is no evidence so far that it does any harm.

Fig. 1 shows that in the lower dose range, increasing the amount of drug infused produced a roughly proportional increase in

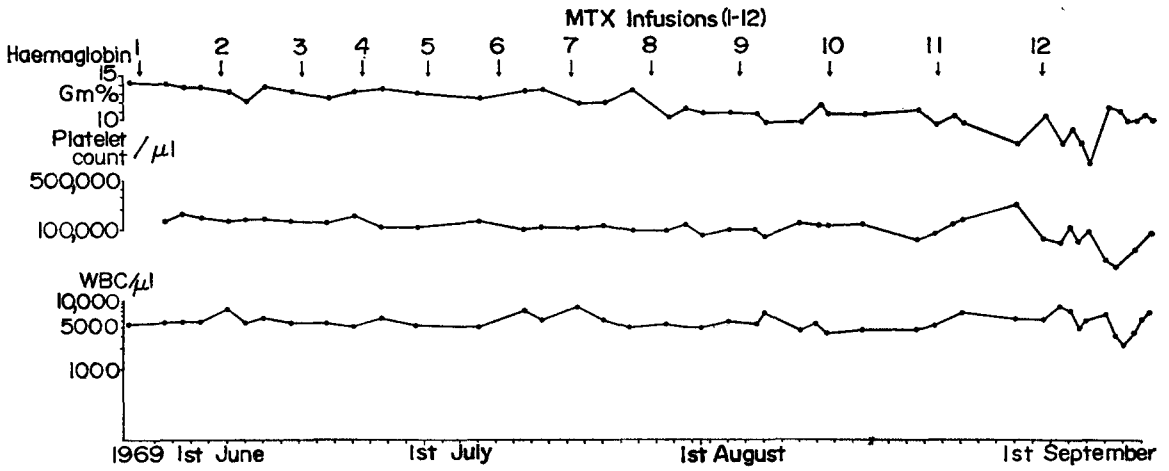

Fig. 3. Peripheral blood chart of patient 1. Note greater fall of Hb, W.B.C. and platelets with infusion 12 (7000 mg over 48 hr) than with infusion 11 (20,000 mg over 24 hr).

plasma concentrations. The 20,000 mg infusion, however, resulted in a plasma concentration far higher than could have been predicted. We are not certain why this disproportionate increase occurred but it is possible that the rate of infusion of the drug exceeded the maximum possible renal excretion rate in this instance.

Patient 7 died 11 days after a 30-hr infusion of 8000 mg. Although his bone marrow at the time of death was reported as normal, both the platelet and leucocyte counts were falling five days after the infusion, and he bled into one of the multiple deposits of melanoma in his lungs 11 days after treatment. This patient's bone marrow reserve had previously been compromised by chemotherapy with vincristine, methotrexate, cyclophosphamide and 5 fluorouracil. In retrospect, we think that insufficient time was allowed for this patient's marrow to recover from previous therapy, and that is was probably unwise to give a 30-hr infusion to begin with. We now begin with short infusions of 12 or 16 hr if there is any doubt at all about the patient's bone marrow reserve, and since adopting this policy we have had no further toxicity. The only other side-effect noted was nausea during two infusions, easily controlled by chlorpromazine. The degree of nausea was again independent of the dose.

We conclude from this study that it is possible to give much larger doses of MTX than are generally employed, provided that the precautions indicated are rigorously observed. An extended study of this kind might enable MTX resistance to be overcome in some instances of malignant disease. In addition, it might theoretically be both safe and more effective to treat non-malignant diseases such as psoriasis by intermittent infusion of smaller doses of say 100 to 200 mg followed by folinic acid, particularly if such infusions were needed only very infrequently. In any event, we believe that the toxicity of MTX can very largely be avoided if the drug is always given over short periods of time as described, irrespective of the dose used.

Acknowledgement—We are very grateful to Dr D. A. G. Galton for his extremely helpful advice and criticism during the preparation of this paper.

ADDENDUM

Since this paper was first written, we have given over 200 infusions of between 200 mg and 400 mg MTX over times varying from 16 to 24 hr, usually in combination with other antitumour drugs, to 50 patients. All the infusions were followed by folinic acid and the precautions described were always observed. No toxicity attributable to MTX has occurred in any of the patients.

Requests for reprints should be addressed to Dr L. A. Price.

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Subcellular Particles in Tumors—V.

Behavior of Mitochondria of Hepatoma HC and Morris Hepatomas 7316A, 7794B and 16 in Density-gradient Centrifugation

S. WATTIAUX-DE CONINCK*, M. COLLOT*, R. WATTIAUX* and H. P. MORRIS†

**Laboratory of Physiological Chemistry, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium and †Department of Biochemistry, Cancer Research Unit, College of Medicine, Howard University, Washington, D.C.*

Abstract—(1) *The distribution of mitochondrial enzymes has been investigated after isopycnic centrifugation of mitochondrial fractions of HC hepatoma and Morris hepatomas* 7794B, 7316A *and* 16. *Two kinds of density gradient were used, one consisting of glycogen dissolved in aqueous sucrose of different molalities and the second of aqueous sucrose alone.*

(2) *In sucrose gradient experiments, the distributions of cytochrome oxidase, monoamine oxidase and glutamate dehydrogenase were established. Isopycnic centrifugation was performed at two speeds,* 39,000 *rev/min and* 65,000 *rev/min. At* 39,000 *rev/min, a similar distribution was seen for the three enzymes in each hepatoma; however, the distribution exhibited marked differences from one hepatoma to the next. The distributions of the three mitochondrial enzymes of hepatomas* 16 *and* HC *remained practically unchanged when centrifugation was performed at* 65,000 *rev/min instead of* 39,000 *rev/min. In contrast, the higher centrifugation speed did induce changes in the distributions of hepatoma* 7316A *and* 7794B *enzymes.*

(3) *In glycogen gradient experiments, cytochrome oxidase distribution was studied. The distribution indicates that the behavior of mitochondria of hepatoma* 7794B, 7316A *and HC in such gradients is qualitatively similar to that of rat liver mitochondria. However, the parameters that characterize the intramitochondrial spaces, calculated from the distribution pattern, differ to a variable extent from those calculated for rat liver particles. The cytochrome oxidase of hepatoma* 16 *behaves as if the mitochondria of this tumor were devoid of an osmotic space.*

INTRODUCTION

THE DISTRIBUTION of cytochrome oxidase, a reference enzyme for mitochondria, after isopycnic centrifugation of rat liver mitochondrial fractions has been studied by Beaufay and Berthet [1] and Beaufay *et al.* [2]. From the behavior of this enzyme in several types of gradients, these authors have concluded that mitochondria are composed of a hydrated matrix and two spaces, one functioning as an osmometer (osmotic space), the other permeable to low molecular weight solutes such as sucrose (sucrose space). We wished to determine to what extent such a model would apply to hepatoma mitochondria. To this end, we investigated the distribution of cytochrome oxidase of transplantable hepatomas of different growth rates after isopycnic centrifugation in glycogen and sucrose gradients. In addition, the distribution in sucrose gradients of two other mitochondrial enzymes, monoamine oxidase and glutamate dehydrogenase, was established. The results presented here were obtained on one rapidly growing tumor (HC hepatoma), two medium-to-slowly growing tumors (Morris

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hepatomas 7794B and 7316A), and on one slowly growing tumor (Morris hepatoma 16).

MATERIAL AND METHODS

Animal

Morris hepatomas 7316A (generations 33, 34, 35, 36, 51], 7794B (generations 13, 14, 15, 16, 21, 23), and 16 (generations 3 and 5) and HC hepatomas (generations 145, 151, 169, 172) were transplanted into Buffalo strain rats at Howard University, Washington D.C.; the tumor-bearing animals were shipped by air express to our laboratory in Namur, Belgium. The average times in days between transplantation and collection of the tumors were: 60 ± 8 for 7316A, 76 ± 13 for 7794B, 176 ± 8 for 16 and 22 ± 3 for HC.

Centrifugations

The rats were killed by decapitation. The tumor was rapidly dissected, cut into small pieces on a refrigerated Petri dish and placed in ice-cold 0·25 M sucrose. The tumor was homogenized and a cytoplasmic extract was prepared as previously described [3]. Mitochondrial fraction was isolated from the extract by one centrifugation at 250,000 $\mathbf{g} \times$ min in the 40 rotor of the Spinco ultracentrifuge followed by one washing. Preliminary fractionation experiments showed that this fraction possesses an activity of cytochrome oxidase, monoamine oxidase and glutamate dehydrogenase corresponding to at least 60 % of the activity found in tumor homogenate. Density gradient experiments were performed according to Beaufay *et al.* [2]. Certain details concerning the experimental procedure are also given in the text.

Enzyme assays

Cytochrome oxidase was measured according to de Duve *et al.* [4], monoamine oxidase according to Schnaitman *et al.* [5] and glutamate dehydrogenase according to Leighton *et al.* [6] except that ADP 0·75 m M was present in the incubation mixture (Tomkins *et al.* [7]).

RESULTS

Sucrose gradient

In these experiments, the mitochondrial preparation is layered at the top of the gradient; during centrifugation, the granules migrate through linearly increasing sucrose concentrations until equilibrium density is attained. Recently, it has been shown that rat liver mitochondria centrifuged in this type of gradient can undergo major changes depending on the speed of centrifugation [8]. First, their equilibrium density increases and the particles behave as if they were growing more permeable to sucrose, after which a true disruption of mitochondrial membranes occurs [8]. These phenomena are readily observed when enzymes of different submitochondrial location are measured.

We investigated the distribution in sucrose gradient of three mitochondrial enzymes: cytochrome oxidase, located in the inner membrane, monoamine oxidase, situated in the outer membrane, and glutamate dehydrogenase, a soluble enzyme of the mitochondrial matrix [9]. Two speeds of centrifugation were used: 39,000 rev/min, a speed similar to that used in glycogen gradient experiments, and 65,000 rev/min. The integral of the square angular velocity $w = \int_0^t \omega^2 \, dt$ was $14{\cdot}4 \times 10^{10}$ $\text{rad}^2 \, \text{sec}^{-1}$ in both cases. Fig. 1 illustrates the distributions observed.

At 39,000 rev/min, a similar distribution was seen for the three enzymes in each hepatoma; however, there were marked differences in distribution from one hepatoma to the next. The distribution curves of hepatoma 16 enzymes were scattered over a narrow density range, with a median equilibrium density of about 1·18. For enzymes of hepatomas 7316A and 7794B, the distribution curves were shifted toward regions of higher density. A bimodal distribution was observed for hepatoma HC enzymes; these were mostly recovered in the bottom of the gradient in a fraction of mean density 1·23, but also exhibited a significant peak of activity in the 1·207 mean density fraction.

The distribution of the three mitochondrial enzymes of hepatomas 16 and HC were virtually unchanged when centrifugation was performed at 65,000 rev/min instead of 39,000 rev/min. On the contrary, marked differences were observed between the distributions of hepatomas 7316A and 7794B enzymes at the two speeds. The most significant change consisted of an extension of the distribution curves toward regions of lower density; moreover, a marked activity peak appeared for cytochrome oxidase and monoamine oxidase in a fraction virtually devoid of glutamate dehydrogenase (fraction of mean density 1·17 for hepatoma 7794B and of mean density 1·16 for hepatoma 7316A).

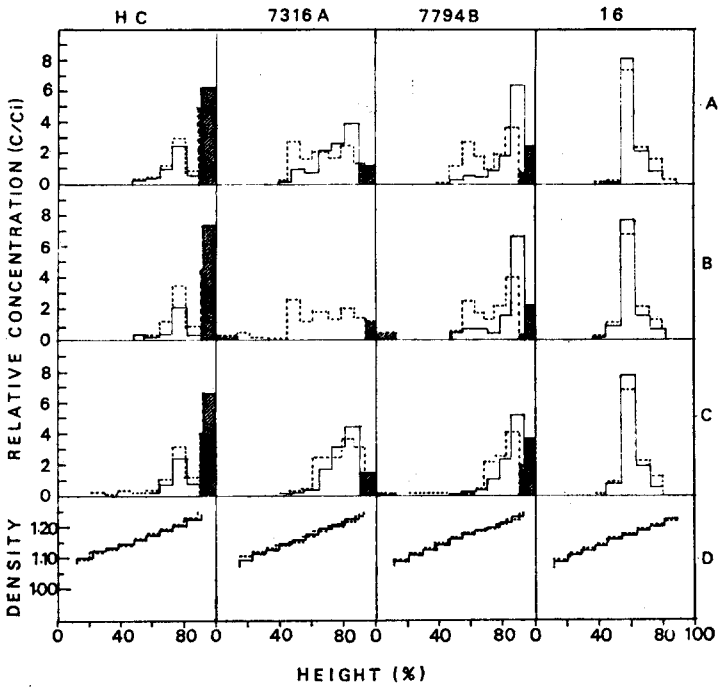

Fig. 1. Distribution patterns of enzymes after centrifugation at 39,000 *rev/min (solid lines) or* 65,000 *rev/min (dotted lines), of a mitochondrial fraction of hepatomas through a* 0·78 *to* 3·42 *molal sucrose gradient in water. The time integral of the square angular velocity* $\int_0^t \omega^2\, dt = 14{\cdot}6 \times 10^{10}$ *rad*^{2} *sec*$^{-1}$. *The particles were initially layered on top of the gradient. Ordinate: relative concentration, i.e. ratio of the observed activity (C) to that which would have been observed if the enzyme had been homogeneously distributed throughout the whole gradient (Ci). Abscissa: percentage of the height of the liquid column in tube. Filled blocks are used for the bottom subfractions to indicate that they include material falling beyond the limits of the gradient. A: cytochrome oxidase; B: monoamine oxidase; C: glutamate dehydrogenase; D: mean density of each subfraction. Recoveries were* 104·7 ± 8·6% *for cytochrome oxidase,* 103·1 ± 7·9% *for monoamine oxidase and* 103·7 ± 13·7% *for glutamate dehydrogenase.*

Glycogen gradient

In these experiments, the granules are homogeneously distributed in the centrifuge tube prior to centrifugation. The gradient consists of glycogen dissolved in a sucrose solution of known molality. Therefore, during centrifugation the subcellular particles are exposed to a constant sucrose concentration. Fig. 2 illustrates the distribution of hepatoma cytochrome oxidase after isopycnic centrifugation in glycogen gradients extending from 0 to 20% with 0·264, 0·561, 0·988 and 1·470 molal sucrose in water as solvent. It is obvious that the distribution of the enzyme and hence of mitochondria is affected by the sucrose concentration of the medium. In order to obtain an average estimation of mitochondrial behavior in the gradient, we calculated the median equilibrium densities of the particles; these are given in Fig. 3. As may be seen, mitochondrial density increases as a function of sucrose concentration but significant differences are apparent among the various hepatomas.

According to Beaufay and Berthet [1], the density of rat liver mitochondria is related to the composition of the suspending medium by the following relationship:

$$\rho_p = \rho_w \frac{\rho_d \alpha + (\rho_d + \rho_m \beta)m}{\rho_d \alpha + \rho_w (1+\beta)m} \qquad (1)$$

where ρ_p is the density of the particle, ρ_d the density of the granule matrix which includes all the solid components of the particle and their hydration water, ρ_m is the density of the medium, ρ_w the density of the solvent (pure water), m the molality of sucrose solution, α the relative particle content of osmotically active solutes, and β the fraction of the particle that is freely accessible to sucrose.

We have calculated the most probable values of α, β and ρ_d using the median equilibrium densities for each hepatoma by the least squares method. Two calculations were performed for hepatomas 7794B, 7316A and 16, one taking into account the medium equilibrium density in sucrose gradient, the second consid-

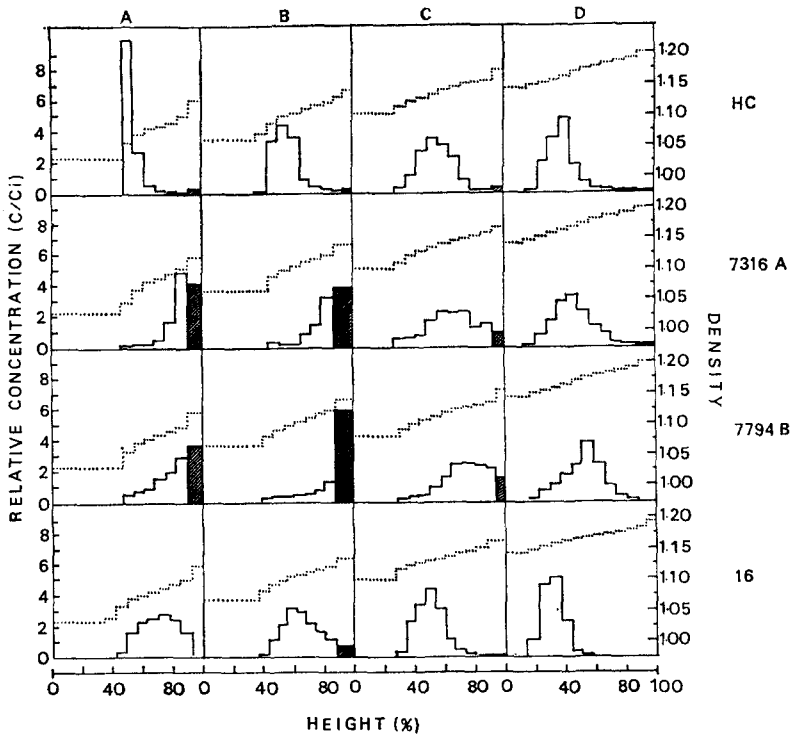

Fig. 2. Distribution patterns of cytochrome oxidase after centrifugation at 39,000 *rev/min* ($\int_0^t \omega^2\, dt = 14{\cdot}6 \times 10^{10}$ *rad*^{2} *sec*$^{-1}$) *of a mitochondrial fraction of hepatoma through a* 0 *to* 20% (*W/V*) *glycogen gradient with* 0·264 (*A*), 0·561 (*B*), 0·988 (*C*) *and* 1·470 (*D*) *molal sucrose in water as solvent. Particles were initially distributed homogeneously throughout the gradient. Dotted line shows the average density of the subfractions. Ordinate: on the left, relative concentration, i.e. ratio, of the observed activity* (*C*) *to that which would have been observed had the enzyme been homogeneously distributed throughout the whole gradient* (*Ci*)*; on the right: density scale. Abscissa: percentage of the height of the liquid column in tube. Filled blocks are used to indicate material falling beyond the limits of the gradient. Average recovery of cytochrome oxidase for the* 16 *experiments was* 93·4 ± 12·5%.

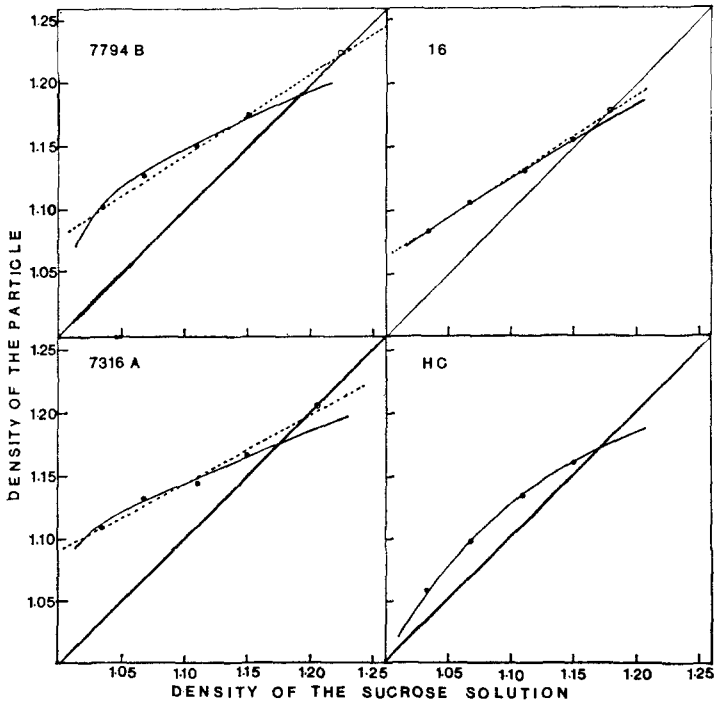

Fig. 3. Median densities of mitochondria as a function of density of sucrose solution. The values were obtained by estimating the median equilibrium densities in glycogen gradients (see Fig. 2). The curves plotted fit equation (a) ——— *or (b)* – – – – *(see text) with the values of* α, β *and* ρ_d *given in Table* 1.

Table 1. *Most probable values for the parameters,* α, β *and* ρ_d *of hepatoma mitochondria.* α *and* β *are given respectively in milliosmoles.* g^{-1} *and* cm^3. cm^{-3} *of hydrated matrix.,* ρ_d *in* $g.cm^{-3}$. (a) *values fitting equation* (1); (b) *values fitting equation* (2) (see text) *The values for the liver are those reported by Beaufay and Berthet* [1]

Hepatoma	HC	7316A		7794B		16		Liver
	(a)	(a)	(b)	(a)	(b)	(a)	(b)	(a)
α	0·716	0·028	0	0·108	0	0·015	0	0·110
β	0·020	0·688	1·16	0·658	1·80	1·49	1·78	0·76
ρ_d	1·26	1·178	1·195	1·203	1·220	1·164	1·171	1·200

ering the equilibrium densities in glycogen gradients only. In the first case, negative values of α were obtained; these are without physical significance. The second type of calculation yielded the α, β and ρ_d values reported in Table 1; the graphs which fit equation (1) with these values are shown in Fig. 3. It will be observed that the curves plotted with these parameters intersect the diagonal at an ordinate value markedly lower than the equilibrium density observed in sucrose gradient. We also attempted to relate the median equilibrium densities of hepatomas 7794B, 7316A and 16 mitochondria to equation (2)

$$\rho_p = \frac{\rho_d + \rho_m \beta}{1 + \beta} \qquad (2)$$

In this case it is supposed that the particle does not exhibit osmotic behavior [1]. The most probable values of β and ρ_d were calculated and the straight lines which fit equation (2) with these values were plotted (Fig. 3). For HC hepatoma mitochondria, only the median equilibrium densities in glycogen gradients were considered since the enzyme distribution in sucrose gradient is bimodal and the median equilibrium density has little significance for this kind of distribution.

DISCUSSION

The results reported in this paper give a general idea of the behavior of the mitochondria of four transplantable hepatomas in density gradients. The modal proposed for rat liver mitochondria applies to hepatoma 7794B and 7316A provided that we discard the median equilibrium density in sucrose gradient. As shown in Fig. 3, this density value largely exceeds the theoretical values obtained on the basis of distributions in glycogen gradients (intersection of the curve with the diagonal). A plausible explanation of this discrepancy is that the mitochondria of hepatomas 7794B and 7316A are altered during centrifugation in the sucrose gradient, even when centrifugation speed does not exceed 39,000 rev/min; they would become more permeable to small solutes such as sucrose which would increase their equilibrium density in sucrose gradient [1]. Such a phenomenon is in fact known to occur for rat liver mitochondria upon centrifugation in a sucrose gradient in a Spinco rotor such as ours but at a speed greater than 39,000 rev/min [8]. The causal agent seems to be the hydrostatic pressure to which the granules are exposed during centrifugation. It is possible that the mitochondria of hepatomas 7794B and 7316A are more fragile than rat liver mitochondria and deteriorate in a sucrose gradient at a centrifugation speed that does not affect rat liver mitochondria. It should be pointed out that the hydrostatic pressure to which mitochondria are subjected during centrifugation in a glycogen gradient, even at the higher sucrose concentration used (1,47 molal), is significantly lower than that to which they are exposed in the sucrose gradient. Another possibility is that the mitochondria of these tumors do not exhibit osmotic behavior and that their density varies linearly as a function of the sucrose concentration of the surrounding medium. However, as shown in Fig. 3, the straight line plotted with the most probable values of β and ρ_d seems not to fit the results obtained in glycogen gradient experiments as closely as the curve plotted according to equation (1).

When isopycnic centrifugation in sucrose gradient is performed at 65,000 rev/min, one observes a change in the distribution of hepatoma 7794B and 7316A mitochondrial enzymes comparable to the changes reported for rat liver mitochondria [8] i.e. a peak of cytochrome oxidase and monoamine oxidase activity appears in a region of the gradient from which only small amount of glutamate dehydrogenase is recovered. This originates from disruption of mitochondria: associated fragments of inner and outer membranes are released from the

granules and equilibrate at a lower density than do intact mitochondria; glutamate dehydrogenase, a soluble enzyme of the mitochondrial matrix, remains at the sites of mitochondrial breakage [8].

The change in hepatoma 16 mitochondria density as a function of sucrose concentration may reasonably be represented by a straight line. These particles behave as if they were devoid of osmotic space. Another peculiarity is that they seem resistant to hydrostatic pressure: an increase of centrifugation speed from 39,000 rev/min to 65,000 rev/min does not affect the distribution of hepatoma 16 mitochondrial enzymes.

The distributions of the three mitochondrial enzymes of hepatoma HC are bimodal after centrifugation at 39,000 rev/min and 65,000 rev/min in sucrose gradient, which suggest that this hepatoma contains two mitochondrial populations. However no bimodality is apparent after centrifugation in glycogen gradients. Again a plausible explanation for the distribution bimodality in sucrose gradient may be that the two kinds of mitochondria are different in their sensitivity to hydrostatic pressure. However, if such is the case, it remains to be explained why a disruption of the granules does not seem to occur when centrifugation speed reaches 65,000 rev/min. It should be pointed out that mitochondria of this rapidly growing hepatoma differ strikingly from the particles of the three other tumors and of the liver in that they exhibit a large osmotic space, a very small sucrose space and an abnormally high hydrated matrix density.

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Action of 1-β-D-Arabinofuranosylcytosine on Mammalian Tumor Cells—2.

Inhibition of Mammalian and Oncogenic Viral Polymerases

WERNER E. G. MÜLLER, ZEN-I. YAMAZAKI, HEINZ H. SÖGTROP and RUDOLF K. ZAHN

Institut für Physiologische Chemie, Johannes Gutenberg Universität, 65 Mainz, Johann Joachim Becher Weg 13, West Germany

Abstract—(1) *1-β-D-Arabinofuranosylcytosinetriphosphate (ara-CTP) is an inhibitor of: DNA-dependent DNA polymerase from mammalian cells, RNA-dependent DNA and DNA-dependent DNA polymerase from Rauscher murine leukemia virus (RMLV) and RNA-dependent DNA polymerase from mouse lymphoma cells.*

(2) *All these inhibitions are of the competitive type. The RNA-dependent DNA polymerases from oncogenic RNA viruses and from lymphoma cells are found to be most sensitive. These enzymes are approximately* 200 *times as sensitive to ara-CTP as DNA-dependent DNA polymerases.*

INTRODUCTION

IT IS known that ara-C inhibits the DNA synthesis of mammalian cells [1, 2], of DNA viruses [3, 4] and of malignant tumors [5–7].

The main target of ara-C is not yet known [review: 14]. In a preceding paper [8] we were able to show that ara-C molecules can be incorporated into DNA of L5178 y mouse lymphoma cells to a relatively large extent without any metabolic alteration. This suggests that drug action may follow as a consequence of this incorporation.

Several investigations explain ara-C action through inhibition of DNA synthesis at the level of DNA-dependent DNA polymerases [survey: 9]. Yet to explain antitumor action of ara-C [10], especially in leukemias [7, 11], other enzymes, specific for oncogenic RNA viruses [12, 13], should be considered [14]. One of the typical enzymes of such viruses [15–17] is RNA-dependent DNA polymerase.

In some cases direct evidence for presence of virus has not been achieved while reverse transcriptase could be demonstrated. Thus Gallo *et al.* [18] and Bosmann [19] have shown human leukemic cells and L5178 y mouse lymphoma cells to contain RNA-dependent DNA polymerases. In view of these facts we discuss in this paper whether antitumor action of ara-C might be caused by inhibition of such an enzyme.

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The abbreviations used are:

ara-C:	1-β-D-arabinofuranosylcytosine
ara-CTP:	1-β-D-arabinofuranosylcytosine-5′-triphosphate
RMLV:	Rauscher murine leukemia virus
K_M:	Michaelis constant
K_I:	Inhibitor constant

MATERIAL AND METHODS

(1) *Source of materials*

The following materials were purchased: unlabeled deoxyribonucleoside triphosphates (dATP, dGTP and dTTP) from Schwarz Bio Research, Orangeburg; ^{3}H-dGTP with a specific activity of 9·1 Ci/mmole from The Radiochemical Centre, Amersham; Nonidet NP 40 from Deutsche Shell Chemie Ges., Hamburg; dithiothreitol from Calbiochem, Los Angeles; micrococcal nuclease and deoxyribonuclease 1 (pancreas) from Worthington Biochemical Corp., Freehold and dialysis tubes (diameter 21 mm) from Visking Comp., Chicago.

Ara-CTP was a gift from Dr. D. Gauchel (Universitätsklinik, Düsseldorf), who synthesized it chemically from ara-C [20]. The sample was chromatographically pure.

The herring DNA was isolated according to Zahn *et al.* [21]. RNA from L 5178 y cells was extracted by a method described by Joel *et al.* [22].

RMLV was obtained from spleens of infected NMRI mice and purified as described before [24]. The L 5178 y mouse lymphoma cells were cultured as described previously [25].

(2) *Isolation of polymerases*

For preparation of crude extracts from mammalian cells a modified method of Scolnick *et al.* [26] was used. Five grams of mammalian cells were pulverized in a mortar together with dry ice, which was ultimately evaporated. The disrupted cells were suspended in 5 ml saturated $(NH_4)_2SO_4$ containing 50 mM Tris-HCl, pH 7·8 and 0·1 mM EDTA and homogenized with 1 g of glass beads (diameter: 0·45–0·55 mm) in a cell homogenizer (type MSK, B. Braun, Melsungen) for 2 min. This preparation was centrifuged at 15 000 × **g** for 1 hr at 2°C. The pellet was resuspended in 5 ml buffer: 50 mM Tris-HCl, pH 7·8, 50 mM KCl and 1 mM dithiothreitol. A diluted solution of "Triton X-100" was added by drops to a final concentration of 0·3 % (v/v). This preparation was kept at 37°C for 15 min and centrifuged at 15 000 × **g** for 1 hr at 2°C. The supernatant was dialyzed for 12 hr at 2°C against 2000 ml buffer 1. The dialyzed extract was then assayed for enzyme activity.

For observing full activities of the RMLV polymerases, the purified virus preparation was preincubated for 10 min at 0°C with 0·2 % Nonidet NP 40 and 0·1 M dithiothreitol.

In all experiments DNA and RNA, resident in the enzyme preparations, were destroyed by a preincubation with micrococcal nuclease [27].

The extracts from mammalian cells contained 3 to 6 mg protein/ml, the virus preparation 0·3 mg protein/ml.

(3) *DNA polymerase assays*

The DNA-dependent DNA polymerase reaction mixture (0·1 ml) contained the following components: 40 mM Tris-HCl (pH 7·8), 60 mM KCl, 2 mM $(CH_3COO)_2Mg$, 2 mM dithiothreitol, 0,1 mM each of dATP, dTTP, ^{3}H-dGTP (5000 cpm/pmole), varying amounts of dCTP and ara-CTP, 20 μg native herring DNA (template saturating conditions) and 10 μl mammalian or RMLV enzyme preparation. The reaction mixture was incubated at 37°C for 15 min. The acid-precipitable radioactivity was collected and counted as previously described [24].

The assay conditions for RNA-dependent DNA polymerase were the same as described for DNA dependent DNA polymerase with the exception that DNA was replaced by RNA. For template saturation 200 μg RNA per ml were used.

(4) *Determination of protein, DNA and RNA*

Protein determinations were performed according to Lowry *et al.* [28]. DNA and RNA were determined by the methods of Kissane *et al.* [23] respectively I-San-Lin *et al.* [29].

(5) *Determination of K_M and K_i*

The calculation of the Michaelis constant of dCTP and the inhibitor constant of ara-CTP was based on double reciprocal plots [30] at two different inhibitor concentrations. Incorporation in the absence of dCTP during the polymerase reaction was subtracted from the data before plotting. In the presence of dATP, dGTP and dTTP, with dCTP missing, incorporation was 15 to 30 % of that obtained in the presence of all four deoxynucleoside triphosphates.

The Michaelis constants and the inhibitor constants (K_M and K_i) for dCTP and ara-CTP were calculated according to Dixon *et al.* [31].

RESULTS

(1) *Determination of Michaelis constants*

The experiments reported in Figs. 1 to 5 show linear relationships for the controls and ara-CTP-inhibited enzyme reactions. The double

reciprocal plot of DNA synthesis versus dCTP concentration shows that the inhibitory action of ara-CTP can be abolished at high dCTP concentrations (Figs. 1 to 5)—the straight lines intersect on the vertical axis—indicating that ara-CTP exerts its effect through competitive enzyme inhibition.

The K_M and the K_i values calculated from the plots (Figs. 1 to 5) correspond to the equilibrium constants for the enzyme substrate and the enzyme inhibitor association reaction. They are presented in Table 1.

(2) *Inhibition of DNA-dependent DNA polymerases*

DNA-dependent DNA polymerases, extracted from three different sources: normal mouse liver, mouse lymphoma cells and RMLV, were tested for ara-CTP inhibition. Their plots are shown in Figs. 1 to 3. The ara-CTP inhibitor constant (Table 1) for the RMLV polymerase is about twice as high as the one for the mammalian enzymes. The Michaelis constant for dCTP of the virus enzyme is three to six times higher than that of the other two DNA-dependent DNA polymerases.

Table 1. *Quantitative comparison of the Michaelis constants of different DNA polymerases for dCTP and ara-CTP*

Enzymes	Source of the enzyme	Michaelis constant for dCTP (μM)	Inhibitor constant for ara-CTP (μM)
DNA-dependent DNA polymerases	Mouse liver	10·2 ± 5·9	10·7 ± 6·7
	Mouse lymphoma cells L 5178 y	21·6 ± 10·5	18·4 ± 7·9
	Rauscher murine leukaemia virus	65·2 ± 31·9	25·7 ± 14·0
RNA-dependent DNA polymerases	Mouse lymphoma cells L 5178 y	15·7 ± 8·5	0·05 ± 0·03
	Rauscher murine leukemia virus	19·3 ± 9·1	0·09 ± 0·05

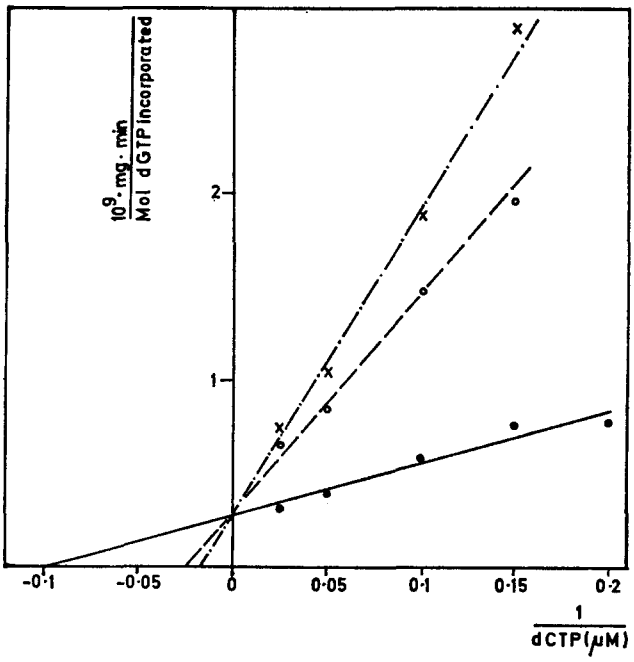

Fig. 1. *Competitive inhibition of DNA-dependent DNA polymerase from normal mouse liver by ara-CTP. Plot according to Lineweaver et al.* [30]. *The reaction conditions were as described in methods. The determination of the K_i of ara-CTP was carried out with* 30 μM *ara-CTP* (○) *and* 60 μM *ara-CTP* (×) *added to the reaction mixture. Control incubation was without ara-CTP* (●). *x-axis: reciprocal values of dCTP concentration; y-axis: reciprocal values of the reaction velocity.*

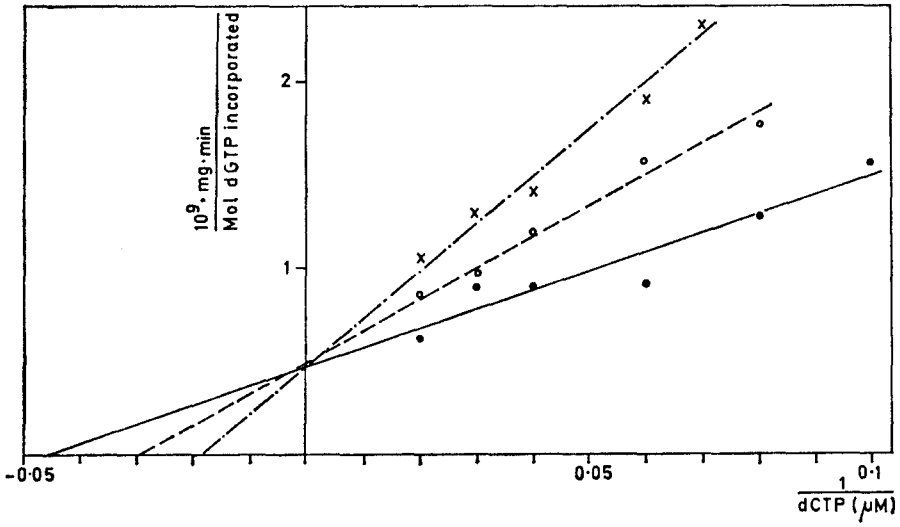

Fig. 2. Competitive inhibition of DNA-dependent DNA polymerase from mouse lymphoma cells L 5178 y. Data for the control reaction (●) and for ara-CTP-inhibited reactions with 10 μM (○) and with 30 μM (×) have been plotted.

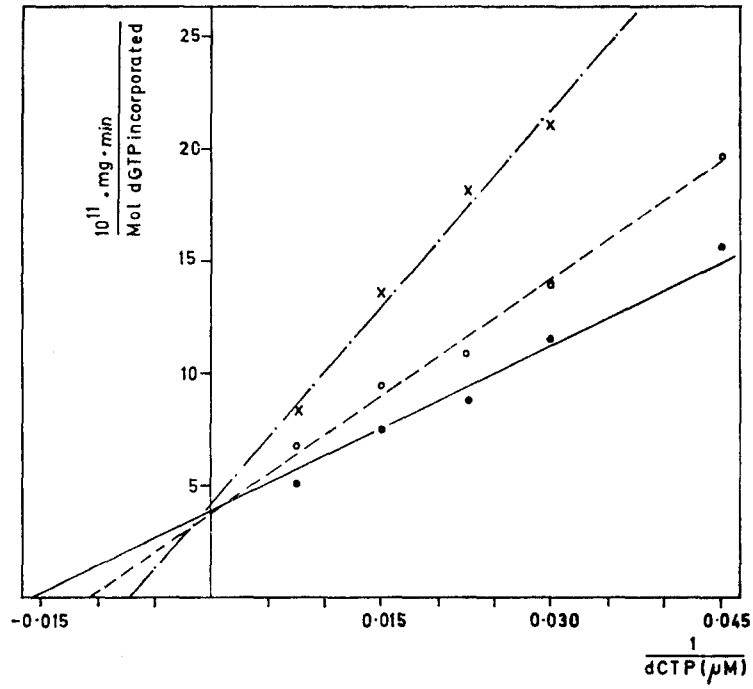

Fig. 3. Competitive inhibition of DNA-dependent DNA polymerase from RMLV. Data for the control reaction (●) and for ara-CTP-inhibited reactions with 10 μM (○) and with 30 μM (×) have been plotted.

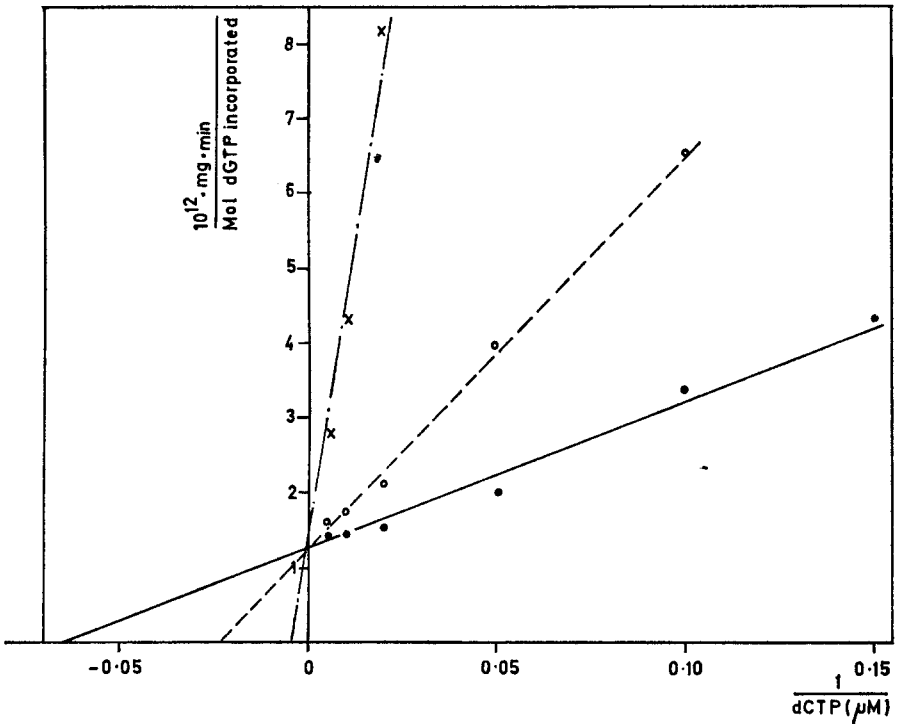

Fig. 4. Competitive inhibition of RNA-dependent DNA polymerase from mouse lymphoma cells L 5178 y. Data for the control reaction (●) and for ara-CTP-inhibited reactions with 0·1 μM (○) and with 0·5 μM (×) have been plotted.

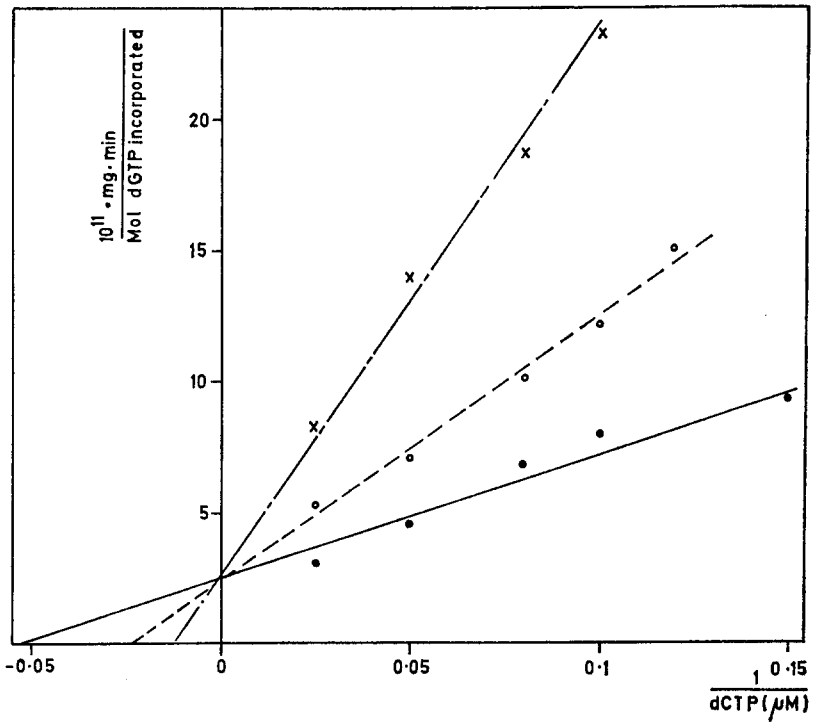

Fig. 5. Competitive inhibition of RNA-dependent DNA polymerase from RMLV. Data for the control reaction (●) and for ara-CTP-inhibited reactions with 0·1 μM (○) and with 0·3 μM (×) have been plotted.

(3) *Inhibition of RNA-dependent DNA polymerases*

The activity of the RNA dependent DNA polymerase from mouse lymphoma cells and from RMLV can be competitively inhibited by ara-CTP also (Figs. 4, 5). The Michaelis constants of these two enzymes for dCTP are in the range (Table 1) of those determined for the mammalian DNA-dependent DNA polymerases. On the other hand a large difference exists between the inhibitor constants of the two classes of polymerases for ara-CTP. The RNA-dependent DNA polymerases are about 200 times as sensitive for ara-CTP as the DNA-dependent DNA polymerases.

In normal mouse liver no RNA-dependent DNA polymerase activity could be detected.

DISCUSSION

While there does not seem to exist any influence of ara-CTP on DNA-dependent RNA polymerases [32–34], DNA-dependent DNA polymerases from mammalian tissues are to a certain extent inhibited, with constants found to agree with Graham *et al.* [33], but higher than those of Furth *et al.* [32]. The RMLV polymerase shows a twice higher inhibitor constant.

In contrast the RMLV RNA-dependent DNA polymerase shows a remarkably high sensitivity towards ara-CTP: it is 200 times as sensitive. This may cause a corresponding susceptibility in RMLV and in mouse lymphoma cells. Since oncogenic viruses [12, 13, 15] as well as mammalian tissues including human ones infected by such viruses are known to contain the enzyme [14], the possibility to inhibit it seems quite interesting.

Though the presence of RNA-dependent DNA polymerase in our L 5178 y cells agrees with the results of Bosmann [19], for more conclusive evidence criteria of Spiegelman *et al.* [35] and of Gallo [14] have been applied by us: (*a*) the dependence of enzyme activity on RNA template, (*b*) the sensitivity of the reaction towards RNase, (*c*) the simultaneous requirement of all four deoxyribonucleoside triphosphates and (*d*) the proof that the product is DNA. In oncogenic RNA viruses a DNA-dependent DNA polymerase is associated with a RNA-dependent DNA polymerase [16]. With the techniques applied in this study it was not possible to detect in extracts from mammalian cells this virus-specific DNA-dependent DNA polymerase. The data given in this paper indicate that the Michaelis constant for dCTP measured in the DNA polymerizing system from mouse lymphoma cells is significantly higher than the value found for the one extracted from mouse liver. The data suggest that this higher constant might be caused by a virus DNA-dependent DNA polymerase possibly coexisting with the host DNA-dependent DNA polymerase of the lymphoma cells, since the Michaelis constant for virus DNA-dependent DNA-polymerase was found to be considerably higher than the one for the host enzyme.

Our results suggest that the RNA-dependent DNA polymerase, which in infection of mammalian cells by oncogenic RNA viruses possibly catalyzes one of the first steps of cell transformation or virus multiplication [14], could be inhibited by quite low concentrations of ara-CTP.

Eidinoff *et al.* [36] have shown that the proliferation of the Rous sarcoma virus, which contains also the RNA-dependent DNA polymerase [13] can be inhibited by ara-C. This can be understood in view of the experiments of Momparler *et al.* [37], who showed that the ara-C molecules taken up by cells are readily phosphorylated to ara-CTP.

On the basis of these results and the data, found in the *in vitro* assays, presented here, it could be concluded that the cytostatical action of ara-C for mammalian cells, infected by oncogenic RNA viruses, is mainly due to a competitive inhibition of the RNA-dependent DNA polymerase by ara-CTP. This conclusion would suppose that the RNA-dependent DNA polymerase has become a necessary enzyme for multiplication of transformed cells.

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Chemotherapeutic 'Fingerprints' of Two Experimental Tumors *in vitro*

L. MORASCA, G. BALCONI, F. DE NADAI, E. DOLFINI

Instituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62–20157 Milano, Italy

Abstract—*Two experimental tumors, (Rhabdomyosarcoma BA* 1112 *growing on rats WAG/Rij and spontaneous mammary carcinoma growing on* $(C_3H \times 020)$ F_1 *mice), were tested* in vitro *under different experimental conditions for their sensitivity to* 10 *antitumoral drugs. The two tumors show a different pattern of sensitivity (chemotherapeutic "fingerprints").*

INTRODUCTION

IN PREVIOUS studies, we had established the sensitivity of human tumors, cultivated in an organotypic culture, to a number of antitumoral drugs [1, 2]

Information of this type may serve a double purpose:

1. Characterization of human tumors in terms of a chemotherapeutic "fingerprint" that could or could not be related to the classification made on a morphological basis;
2. Choice of the best treatment for the patient.

In order to gain a better understanding of the problems involved in this investigation, it was decided to also utilize animal models which allow for more quantitative and controlled studies.

Two experimental tumors were thus selected, namely, Rhabdomyosarcoma BA 1112 [3] and a spontaneous mammary carcinoma [4, 5].

The rhabdomyosarcoma is transplantable in the isogenic strain WAG/Rij and it is available also as a tissue culture line. The mammary tumor develops spontaneously in $(C_3H \times 020)F_1$ female mice by forced breeding, and in castrated males, treated with estrogens.

These two tumors have been employed to compare the response of *in vitro* cultures to antitumoral agents, in various conditions, and to assess the reproducibility of the results with tumors spontaneously developed and grown in different subjects of the same strain.

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MATERIAL AND METHODS

Tumors

Rhabdomyosarcoma BA 1112 was isolated from the mandibula of a radiation chimera of the WAG/Rij inbred strain of rats [3]. This tumor is carried as a transplantable line in the isologous strain of these animals. By a trypsinization procedure described by Reinhold [6], the tumor cells can be grown *in vitro* where they behave as a cell line. The *in vitro* line is able to produce tumors, when it is transplanted in WAG/Rij rats. In our experiments, the *in vitro* line was carried through several weekly transplantations and stored in liquid nitrogen, before performing the experiments. The *in vivo* line was tested *in vitro* by the Rose Chamber explant method [7], taking biopsy material directly from the animal.

The mammary tumor of the $(C_3H \times 020)F_1$ mice appears spontaneously in almost 100% of force-bred females and in castrated males treated with estrogens [5]. These tumors were obtained from the International Center for the Provision and Study of Tumor-Bearing Animals, Amsterdam (Prof. O. Mühlbock).

Culture media

Eagle's MEM on Hank's base, obtained from

GIBCO (Grand Island, N.Y.) as a dehydrated preparation, was used throughout all the experiments.

This growth medium was supplemented by 10% calf serum.

Trypsin was obtained from Difco (Detroit Mich., U.S.A.). Working solutions were 0·25% in Ca- and Mg-free Hank's solution.

CITOTOXICITY STUDIES

Rotating cultures

A cell suspension, containing 10^5 Rhabdomyosarcoma BA 1112 cells/ml, was distributed in test tubes placed on a slow-rotating roller drum and incubated for 48 hr following our standard method [8].

The number of cells released in suspension by a treatment with trypsin, was measured by electronic evaluation (Coulter Counter mod. B) on groups of five test tubes (Base line). The remaining tubes were then treated with serial dilutions of different drugs, and the number of cells was again evaluated after 24 hr incubation.

The results are expressed as a percentage of inhibition of the growth of controls, and as a percentage of cell destruction in relation to the base line. Each point represents the mean of five samples $\pm$S.E.

Stationary cultures

The same method described under "Rotating Cultures" was applied to cultures of Rhabdomyosarcoma BA 1112 growing in Leighton's flat tubes in stationary conditions.

Explants in Rose's Chambers

WAG/Rij male rats, bearing Rhabdomyosarcoma BA 1112, and $(CH_3 \times 020)F_1$ male and female mice, bearing a spontaneous mammary carcinoma, were killed by ether anesthesia and the neoplastic tissues were aseptically removed.

Fifty to seventy Rose's Chambers were prepared from each animal. In a first group of experiments, each drug was tested in a wide range of doses that were then restricted to the doses closest to the threshold of cytotoxicity.

Explants of neoplastic tissue of about one mm were cut with sharp blades and implanted in Rose's Chambers by the perforated cellophane technique [7].

Incubation was performed in a CO_2 incubator at about 37°C. Cultures were scored daily by phase-contrast microscopy until the first outgrowth was detectable. The morphology was typical for each tumor: rhabdomyosarcoma BA 1112 gave a population of spindle-shaped cells, extremely regular in their morphological aspects; the mammary tumor resulted in a mixed population containing epithelial cells mingled with bundles of fibroblasts and a few endothelia.

Treatment was started immediately after the outgrowth was detected, and the microscopic field containing cells was recorded by phase-contrast photography. Pictures were taken at 100 × on a 35 mm film and the observed field was marked on the outside of the coverslip by India ink. The same field was recorded again after 24 hr of treatment by phase-contrast photography.

The "Lethal Endpoint" (LE) was established as the minimal dose capable of destroying the cancer cell population after 24 hr.

In experiments performed on the mammary carcinoma, a "Cytotoxic Endpoint" (CE) was defined as the lowest dose able to induce toxic morphological alterations.

Drugs

Drugs were obtained by courtesy of the following suppliers: methyl-bis-chloroethylamine, DL-sarcolysine, triethylenemelamine (TEM) and triethyleneiminobenzoquinone (Trenimon) from Simes, Milan, Italy. Mannitol mustard from Chinoin, Budapest, Hungary. Cyclophosphamide from Asta Werke, Brackwede, German Federal Republic. Mitomycin C, from Kyowa, Japan. Actinomycin D from Merck, Sharp and Dohme, Rahway, N.J., U.S.A.; 5-fluorouracil from Hoffman La Roche, Basel, Switzerland. Amethopterine from Lederle, Pearl River, U.S.A.

RESULTS AND DISCUSSION

Effect of antitumoral drugs on Rhabdomyosarcoma BA 1112 cultivated in different experimental conditions

Charts 1 to 10 report the effect of ten antitumoral drugs on the growth of rhabdomyosarcoma BA 1112 cells cultivated as rotating or stationary cultures. All the drugs, with the exception of cyclophosphamide, amethopterine and 5-fluorouracil, are able to inhibit growth at lower doses and to destroy cancer cells at higher doses.

5-fluorouracil and amethopterine are, however, able to inhibit growth without destroying the cancer cells. The stationary cultures are definitely more sensitive than the rotating cultures to methyl-bis-(chloroethylamine), mannitol mustard, triethylenemelamine, Trenimon, mitomycin C, and amethopterine.

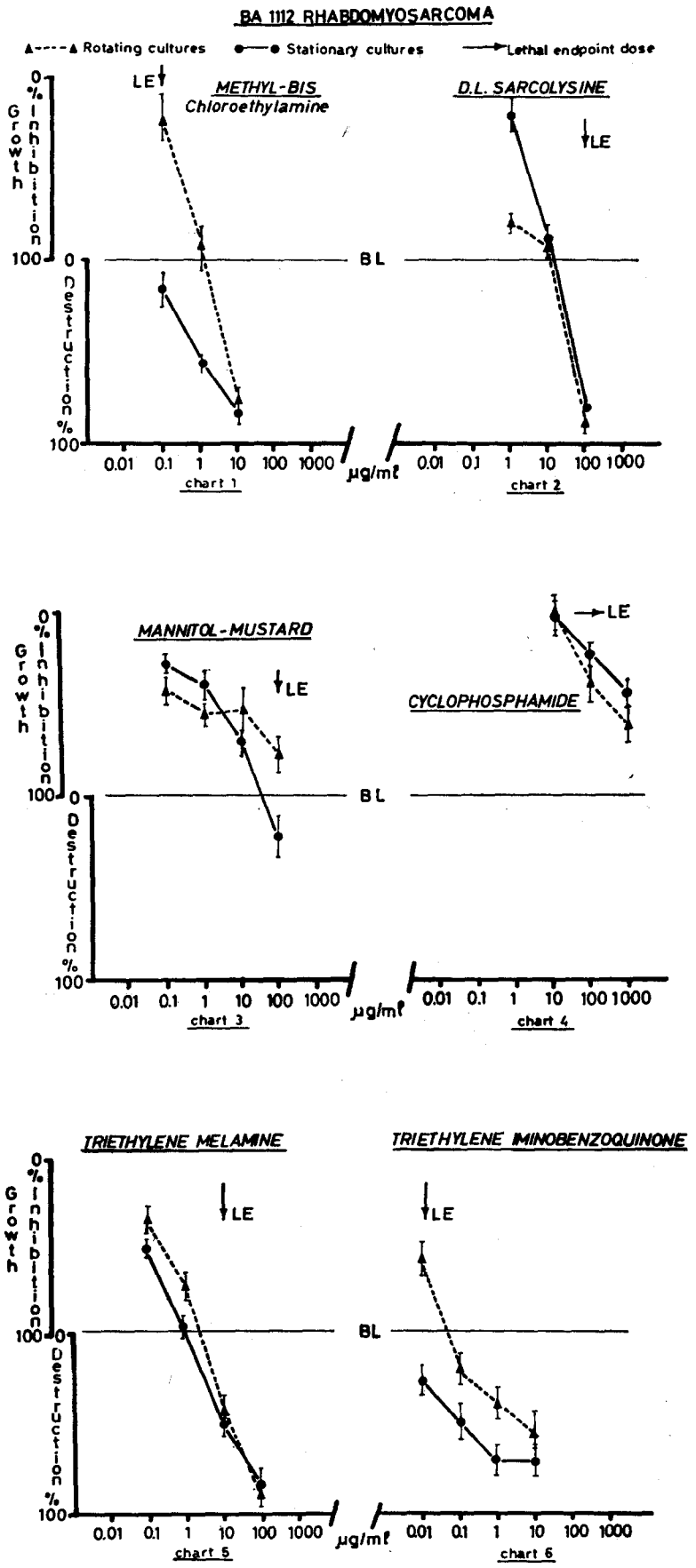

Fig. 1. Sensitivity of rhabdomyosarcoma BA 1112 to various antitumoral agents. ↓ LE refers to the concentration showing lethal effects on explants cultivated in Rose's Chambers.

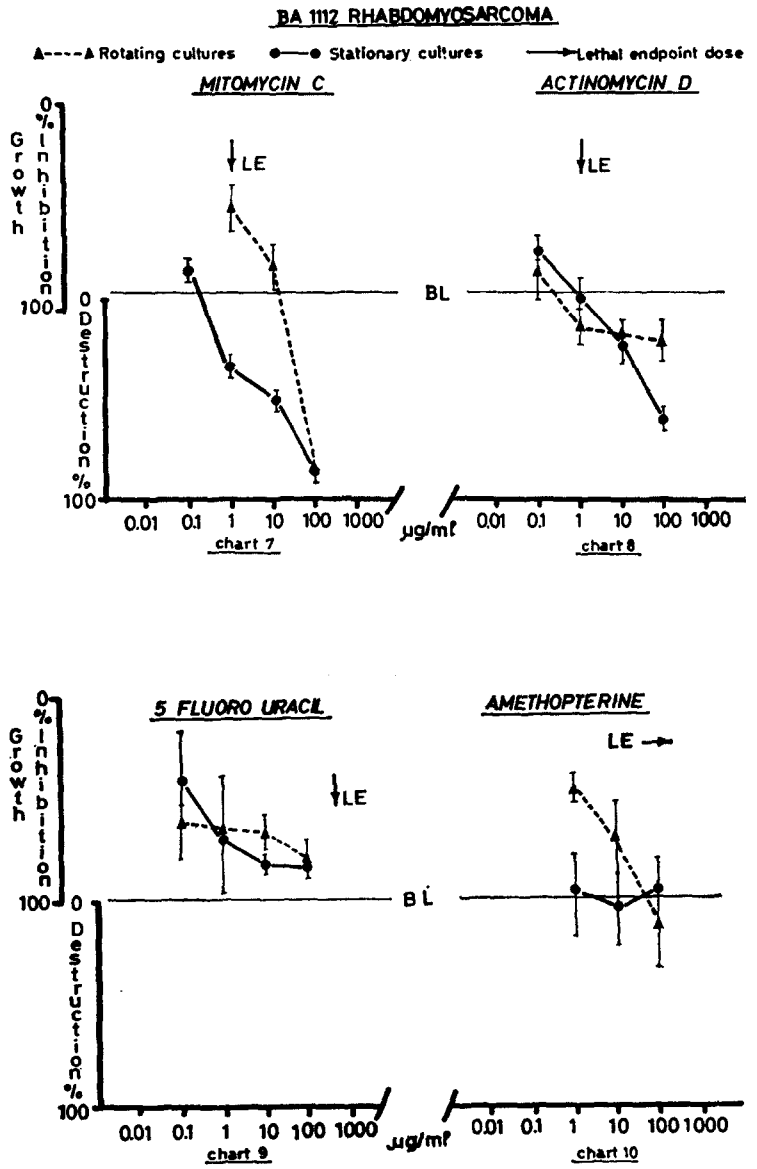

Fig. 2. Sensitivity of rhabdomyosarcoma BA 1112 *to various antitumoral agents. LE* ↓ *refers to the concentration showing lethal effects on explants cultivated in Rose's Chambers.*

These results are summarized in Table 1 which gives also a comparison of the sensitivity of the explants to the same antitumoral drugs. Although the range of activity of the various drugs for the three types of tissue culture is comparable, the explants appear less sensitive than the stationary cultures. This is expected because of the two different levels of toxicity measured by the two methods, namely LE and total growth inhibition. On the other hand, it was not possible for methodological reasons, to establish a LE in the experiments with rotating and stationary cultures.

Rotating cultures are anyhow still less sensitive than explant cultures to methyl-bis-(chloroethylamine), mannitol mustard, triethyleneiminobenzoquinone, and mitomycin C.

In the case of DL-sarcolysine, triethylenemelamine, actinomycin D, and amethopterine, rotating cultures prove to be more sensitive than explant cultures, as is to be expected.

Effect of antitumoral drugs on spontaneous mammary carcinoma explants obtained from different ($C_3H \times 020$) F_1 *mice*

Table 2 summarizes the results obtained by evaluating the sensitivity of the explants to the cytotoxic or lethal effects of the drugs under study. As expected, the lethal effect usually

Table 1. *Effect of antitumoral drugs on Rhabdomyosarcoma BA* 1112 *cultivated* in vitro *under different conditions*

Antitumoral agent	Explants lethal dose (μg/ml)	*In vitro* line inhibiting dose* (μg/ml)	
		Stationary cultures	Rotating cultures
1 Methyl-bis-(chloroethylamine)	0·1	<0·1	1
2 DL-Sarcolysine	100	10	10
3 Mannitol Mustard	100	50	> 100
4 Cyclophosphamide	> 1000	> 1000	> 1000
5 Triethylenemelamine	10	1	5
6 Triethyleneiminobenzoquinone	0·01	< 0·01	0·05
7 Mitomycin C	1	0·1	10
8 Actinomycin D	1	1	0·5
9 5-Fluorouracil	1000	> 100	> 100
10 Amethopterine	> 1000	1	50

*Extrapolated from the charts (80–100% inhibition).

Table 2. In vitro *sensitivity of spontaneous mammary carcinoma explants to various antitumoral agents*

Antitumoral agent	Response at the concentration of (μg/ml)						
	0·01	0·1	1	10	100	1000	
1 Methyl-bis-(chloroethylamine)		0/2	0/4	0/4	2/2		(CE)
		0/2	0/4	0/4	2/2		(LE)
2 DL-Sarcolysine			0/3	2/4	5/5		(CE)
			0/3	0/4	4/5		(LE)
3 Mannitol mustard			0/2	1/3	3/3		(CE)
			0/2	0/3	3/3		(LE)
4 Cyclophosphamide			0/3	0/4	0/4	0/5	(CE)
			0/3	0/4	0/4	0/5	(LE)
5 Triethylenemelamine			0/3	3/5	4/4		(CE)
			0/3	1/4	3/5		(LE)
6 Triethyleneiminobenzoquinone	3/3	4/4	2/2	1/1			(CE)
	3/3	4/4	2/2	1/1			(LE)
7 Mitomycin C			0/2	4/4	4/4		(CE)
			0/2	0/4	4/4		(LE)
8 Actinomycin D	0/4	6/6	6/6	8/8	4/4		(CE)
	0/4	3/6	5/6	8/8	4/4		(LE)
9 5-Fluorouracil			0/2	1/3	3/3		(CE)
			0/2	0/3	0/3		(LE)
10 Amethopterine			0/4	0/4	0/2	2/4	(CE)
			0/4	0/4	0/2	0/4	(LE)

(CE) Cytotoxic effect
(LE) Lethal effect
The figure indicates the number of explants showing CE or LE in relation to the number of cultures treated with a given concentration of the drug.

Table 3. Comparison of the lethal effect of various antitumoral drugs for explants of rat Rhabdomyosarcoma BA 1112 and mouse spontaneous mammary carcinoma

Antitumoral agent	Lethal dose (μg/ml) for explant cultures	
	Rhabdomyosarcoma BA 1112	Spontaneous mammary carcinoma
1 Methyl-bis-(chloroethylamine)	0·1	10
2 DL-Sarcolysine	100	100
3 Mannitol mustard	100	100
4 Cyclophosphamide	> 1000	> 1000
5 Triethylenemelamine	10	100
6 Triethyleneiminobenzoquinone	0·01	< 0·01
7 Mitomycine C	1	100
8 Actinomycin D	1	0·1
9 5-Fluorouracil	1000	> 100
10 Amethopterine	1000	> 1000

occurs at higher concentrations than the cytotoxic one.

In most of the cases, there is a dose–response relationship and a good reproducibility of results.

Trenimon and actinomycin D were the most effective compounds, while cyclophosphamide was inactive, as anticipated, even at the concentration of 1 mg/ml.

Chemotherapeutic "fingerprint" of the two experimental tumors

Table 3 compares the minimal concentration of several antitumoral drugs exerting a lethal effect on rhabdomyosarcoma BA 1112 and on mammary carcinoma. The pattern of sensitivity of the two tumors was quite similar, with two remarkable exceptions: rhabdomyosarcoma was 100 times more sensitive to methyl-bis-(chloroethylamine) and to mitomycin C, compared to spontaneous mammary carcinoma.

On the other hand, mammary carcinoma appeared more sensitive to actinomycin D than did rhabdomyosarcoma.

CONCLUSION

The experiments reported here constitute a part of a more extensive study aimed at obtaining the chemotherapeutic "fingerprints" of various experimental and human tumors, cultivated as primary organotypic cultures. Since tumors are made up of a mixed population of normal and neoplastic cells, only morphological criteria can be utilized for assessing the specific activity of antitumoral drugs. For this reason it is, however, difficult to apply precise quantitative criteria. Despite these limitations, the following conclusions may be made:

1. Studies on Rhabdomyosarcoma BA 1112 indicate that cells from an *in vitro* line and from explants in primary culture exhibit a similar sensitivity to antitumoral agents, although different parameters were used to detect toxicity.
2. It appears that the response of cancer cells *in vitro* may be altered by environmental conditions as in the case of rotating cultures.
3. Investigations on spontaneous mammary carcinoma indicate that tumors of similar origin and structure, growing in different individuals, display a fairly reproducible pattern of sensitivity to a number of antitumoral drugs.
4. The explants obtained from two types of tumors, Rhabdomyosarcoma BA 1112 and mammary carcinoma from $(C_3H \times 020)F_1$ mice, show a pattern of sensitivity to antitumoral drugs which differs in several respects.

These studies provide important background data for the characterization of biopsied human tumors, in terms of their sensitivity to antitumoral agents. As far as the significance of these findings is concerned for predicting the *in vivo* sensitivity of the tumors to antitumoral agents, it is very difficult to make any extrapolation without proper knowledge of pharmacokinetic parameters for the various antitumoral agents.

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Metabolic Controls in Precancerous Liver—II.

Loss of Feedback Control of Cholesterol Synthesis, Measured Repeatedly *in vivo*, During Treatment with the Carcinogens N-2-Fluorenylacetamide and Aflatoxin

BRIAN J. HORTON, JEANETTE D. HORTON and JOHN R. SABINE

Department of Animal Physiology, Waite Agricultural Research Institute, University of Adelaide, South Australia, Australia

Abstract—*We have examined feedback control of cholesterogenesis in rat liver during the course of carcinogen treatment, using an assay for cholesterol synthesis which permits repeated tests on the same animal. Rats were treated with AY-9944 (which inhibits the conversion of 7-dehydrocholesterol to cholesterol), and then 24 hr later blood levels of 7-dehydrocholesterol were measured. This gives an accurate estimation of liver sterol synthesis during that 24 hr period. By feeding 5% cholesterol for two days before each alternate assay, it was possible to measure feedback control of cholesterol synthesis for each individual animal during the carcinogen treatment.*

Rats injected with a single LD_{50} dose of aflatoxin showed a severe loss of control of cholesterogenesis 3 to 5 weeks later. Most animals had recovered normal control 10 weeks after the aflatoxin treatment.

N-2-fluorenylacetamide feeding caused a similar marked loss of control within 1 to 2 weeks, and here also most animals regained normal control later.

With both carcinogens the actual degree of loss of control of cholesterol synthesis was extremely variable between individual animals. Only a few animals showed complete loss of control and there was a wide range of varying degrees of partial control.

Control of cholesterogenesis, as measured by two entirely independent assay systems, has now been shown to be defective after treatment with two different carcinogens, thus confirming the importance in the carcinogenic process, at least in the liver, of this loss of control previously known to occur in all fully-developed hepatomas.

INTRODUCTION

EVERY fully-developed hepatoma so far examined, whether primary or transplanted, in rat, mouse and man, has displayed defective "feedback"* control of cholesterol synthesis [1]. Moreover, this control is also missing from "precancerous" livers, i.e. livers of animals either genetically-prone to develop hepatomas [2] or that have been treated with hepatocarcinogens [3–6].

In a recent paper [5] we reported that feeding rats the carcinogen N-2-fluorenylacetamide (FAA) disrupted both normal endogenous control and also feedback control of hepatic cholesterol synthesis within 2 to 3 weeks. In

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*Inhibition of cholesterol synthesis by cholesterol feeding is often referred to as "feedback" inhibition [2] We use this term in a general sense only, and do not intend to imply any particular mechanism of action.

that study cholesterol synthesis was measured in tissue slices, which meant that each animal had to be killed to carry out the assay. Thus it was not possible to study whether loss of control was related directly to tumour development, nor to measure the exact time course of loss of control, nor to determine whether loss of control was reversible or irreversible for any individual animal (though studies *in vitro* [5, 6] had suggested that control, once lost, could be regained). In order to make these determinations it is necessary to carry out repeated assays for liver cholesterol synthesis on the same animals during the course of carcinogenesis. We have recently developed a vival* assay which makes this possible [8].

This assay depends on the fact that, in the rat, the liver is the major source of serum cholesterol. If rats are treated with AY-9944,† which inhibits the conversion of 7-dehydrocholesterol to cholesterol [9], 7-dehydrocholesterol is found in the serum [10] and the level of this is representative of the cholesterol which would have been synthesized and released into the plasma had AY-9944 not been present [9]. Although this assay actually measures total endogenous sterol production and release into the plasma we have shown that, at least in the rat, it also gives an accurate estimation of liver sterol synthesis [8].

In the present study we have used this vival assay to study feedback control of cholesterol synthesis after a single LD_{50} dose of aflatoxin, and during FAA feeding. A preliminary report of part of this work has already appeared [11].

MATERIALS AND METHODS

Animals and diets

Male, hooded-Wistar rats were used, aged 2 to 3 months at the beginning of each experiment. All animals were housed in a windowless but well-ventilated room (21 to 26°C) with lighting strictly controlled; on at 6 a.m., off at 6 p.m. They were fed a commercial laboratory ration *ad libitum* (Charlicks M164 mouse cubes, either powdered or as cubes). The cholesterol content of this feed was $0{\cdot}07 \pm 0{\cdot}01$ %, measured as digitonin-precipitable sterols. This was supplemented, where indicated, with 0·05% FAA (K & K Laboratories Inc., Plainview, N.J.), fed according to the following schedule—3 weeks FAA, 1 week chow, 2 weeks FAA, 2 weeks chow, 3 weeks FAA, 5 weeks chow [12].

The aflatoxin-treated rats were given 5 mg/kg of aflatoxin, dissolved in dimethylsulfoxide (1 ml/kg), injected intraperitoneally. The aflatoxin was a mixture containing approximately 30% B_1 and 60% G_1, a gift from the British Medical Research Council.

Vival assay for liver cholesterogenesis

We have described this assay in detail previously [8]. Briefly, rats were given 10 μmole/kg AY-9944 (the gift of Dr. Morris Givner, of Ayerst Laboratories, Montreal, Canada). Twenty-four hours later blood was collected from the tail vein, saponified, and then extracted with petroleum spirit (b.p. 40–60°C). After evaporation of the petroleum spirit, 7-dehydrocholesterol was measured as "fast acting sterols", by the colour development at 625 mμ exactly 2 min after the addition of stable Liebermann-Burchard reagent [13].

For 2 days, before and including each test period, the diet of half the animals was supplemented with 5% cholesterol (American Cholesterol Products, Inc., Edison, N.J.). At the following test period the other half were fed 5% cholesterol. Thus for each animal, each assay measured cholesterol synthesis while it was receiving alternately a low-cholesterol and a high-cholesterol diet.

RESULTS

Aflatoxin

Thirty rats were injected (i.p.) with 5 mg/kg aflatoxin dissolved in dimethylsulfoxide and 6 controls with dimethylsulfoxide alone (1 ml/kg). Eleven rats died from the toxic effects of the aflatoxin. The surviving animals were divided into two groups, A and B, which were tested for cholesterol synthesis 1½, 2½, 3½, 5, 7, 10 and 18 weeks after the aflatoxin treatment. Each group was fed 5% cholesterol for 2 days at each alternate assay (i.e. group A was fed cholesterol at 1½, 3½, 7 and 18 weeks and group B at 2½, 5 and 10 weeks).

Figure 1 shows the percentage inhibition of cholesterol synthesis caused by cholesterol feeding. This inhibition was calculated for each animal by comparing its synthesis while on the 5% cholesterol diet with synthesis measured in the previous assay and the following assay (i.e. on the low-cholesterol diet). The control animals consistently gave about 80% inhibition, while the aflatoxin-treated rats showed a marked loss of control, particularly at 3½ and 5 weeks. The percentage

*Green has suggested [7] that the terms "vival" and "vitral" should be used in place of *in vivo* and *in vitro* when these are required as adjectives.

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

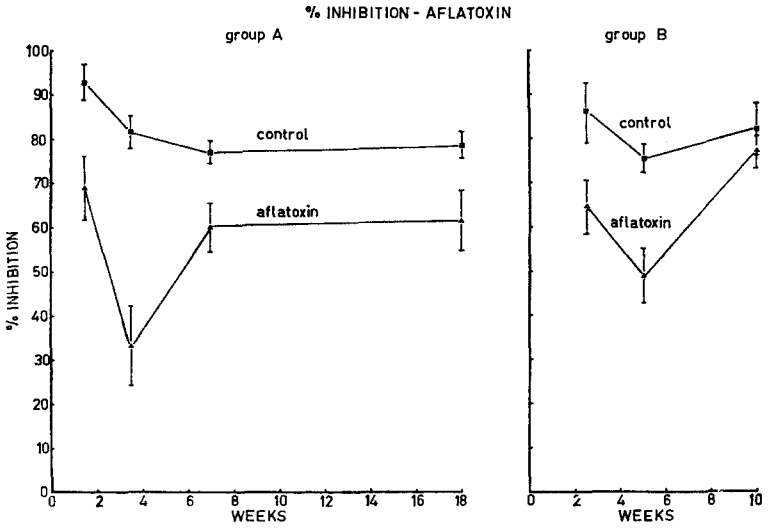

Fig. 1. *Mean* (±*S.E.*) % *inhibition of sterol synthesis by* 5% *cholesterol feeding for* 2 *days in control and aflatoxin-treated rats.* % *inhibition was determined by comparing synthesis while on the* 5% *cholesterol diet with synthesis measured in the previous assay and the following assay* (*i.e. on the low-cholesterol diet*). *Each group contained* 3 *control rats and* 11 (*Group A*) *or* 8 (*Group B*) *aflatoxin-treated rats.*

inhibition for the treated animals was significantly less than that for the controls ($P < 0{\cdot}05$) at all times tested up to and including 7 weeks.

In those animals receiving the low-cholesterol diet aflatoxin caused an increase in cholesterol synthesis. At any given time the difference between the controls and the aflatoxin-treated group was not statistically significant, but when the results for $2\frac{1}{2}$ to 7 weeks were combined the increase in synthesis was significant ($P < 0{\cdot}05$, Mann-Whitney "U" test). This increase is consistent with our previous findings [5] of elevated cholesterogenesis following FAA-feeding, and we have interpreted this as a disruption of normal, endogenous, feedback control [5].

N-2-fluorenylacetamide

Rats were fed FAA according to the schedule, outlined in Materials and Methods, which was reported by Epstein *et al.* [12] to produce large, hyperplastic liver nodules in 50% of rats after 15 weeks, and hepatomas in 80% after 10 months. Under our conditions, however, only 3/23 rats had actually developed hyperplastic liver nodules when examined by laparotomy at 15 weeks. In the presentation of our results for the rats fed FAA, those which did develop hyperplastic nodules have been shown separately from those that did not. The test and control rats were divided into two groups in a manner similar to that in the aflatoxin experiment. Group C was fed cholesterol at 1, 3, 5, 8 and 14 weeks and Group D at 2, 4, 6 and 11 weeks.

Figure 2 shows the percentage inhibition of cholesterol synthesis by cholesterol feeding. The control rats generally had about 80% inhibition of cholesterol synthesis, except for group D at 4 and 6 weeks. The FAA-fed rats, however, showed significantly less inhibition, i.e. a loss of control, and those which ultimately developed hyperplastic nodules showed the greatest loss of all. In fact, all 3 of these nodular rats had lost control completely after either 1 or 3 weeks FAA feeding, though they partially recovered later. Only one other rat showed complete loss of feedback control at this time. The loss of feedback inhibition by the FAA-fed rats was statistically significant ($P < 0{\cdot}05$) at 1, 2, 3, 5, 6, 8 and 11 weeks.

And again, in those rats receiving the low-cholesterol diet, cholesterogenesis was increased by carcinogen treatment. This increase was significant ($P < 0{\cdot}05$) at 2, 5, 6 and 14 weeks. Among the FAA-fed animals those which ultimately developed nodules tended to have higher cholesterol synthesis than those which did not, though this difference was not statistically significant.

Individual variation

Mean values for inhibition, as shown in Figs. 1 and 2, can be misleading because of wide variation between individual, carcinogen-treated animals. Such variation at any given

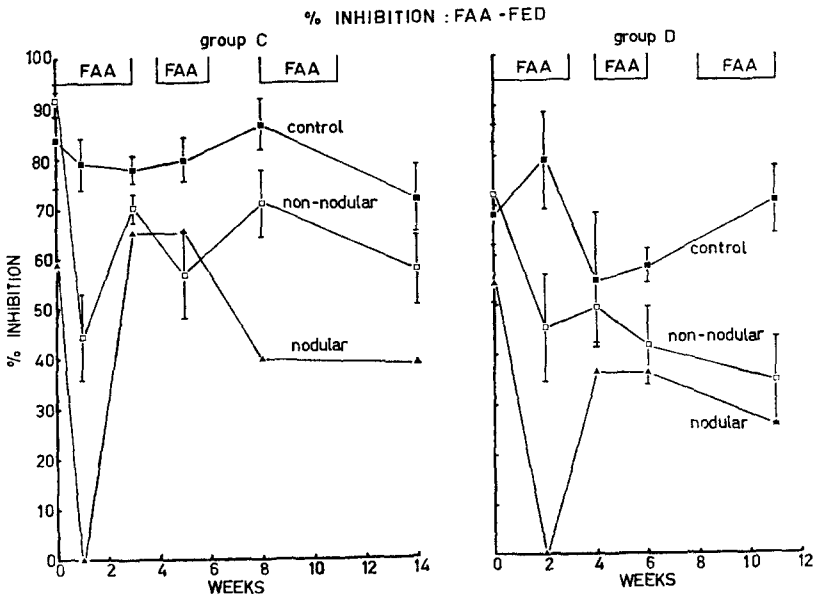

Fig. 2. Mean % inhibition of sterol synthesis (determined as for Fig. 1) in control and FAA-fed rats. Each group contained 4 control rats, 11 (Group C) or 9 (Group D) rats fed FAA but which did not develop hyperplastic nodules by 15 weeks, and 1 (Group C) or 2 (Group D) rats which were fed FAA and did develop nodules. The S.E. is shown for all points except those representing the nodular animals.

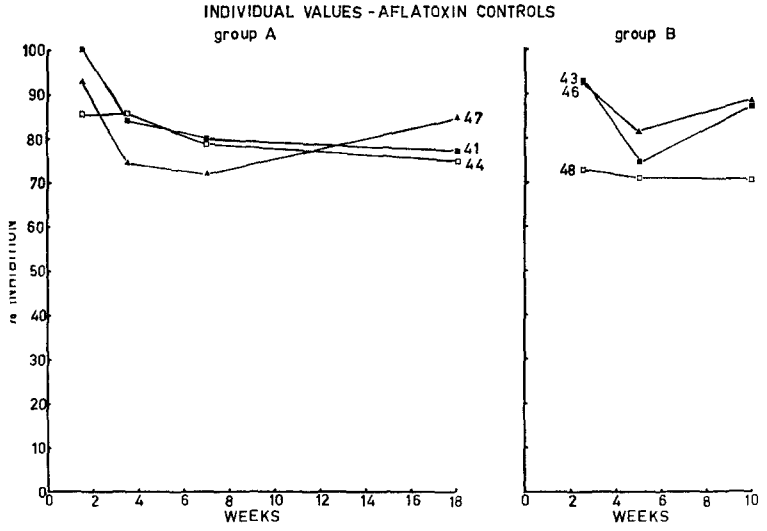

Fig. 3. Individual values for % inhibition of sterol synthesis for the aflatoxin control rats.

time during FAA feeding has already been reported in the vitral studies presented previously [5], but the present results indicate that significant variation also occurs from week to week in each individual animal.

Individual values for the control animals are presented in Figs. 3 and 4. These rats were relatively consistent in their degree of control, except for one (no. 18) in group D. We have since observed (unpublished observations) that in our young rats (2–3 months) about 10 % show poor control, but all show the normal pattern by 4–5 months.

Individual values for several of the aflatoxin-treated and FAA-fed animals are shown in Figs. 5 and 6. The extensive variation seen in these carcinogen-treated animals is far greater than can be explained by the occasional odd one like no. 18. Most rats lost control to some extent within the first 2 weeks, but with considerable variation in the extent of the loss. After the first 2 weeks, however, some regained control permanently (Fig. 5, nos. 4, 12; Fig. 6, no. 34), others regained control but lost it again later (Fig. 5, no. 2; Fig. 6, nos. 8, 27) and in others the control became worse before improv-

ing (Fig. 5, no. 24; Fig. 6, no. 36). This individual variation in loss and recovery of control appeared to bear no relation to the interruption in FAA feeding at 4 and 7 weeks.

DISCUSSION

We have reported previously that FAA feeding and aflatoxin injection cause severe alterations in control of cholesterol synthesis, as measured *in vitro* [5, 6]. Cholesterol synthesis was extremely variable, though generally increased, and feedback control was either partially or completely lost. The vival study presented in this paper has confirmed these observations and extended them by allowing a study of the time course of variation within each individual animal. Study of the actual time sequence of loss of control for individual animals is important, since it is apparent that complete loss of control and almost complete recovery can occur in a much shorter time than the lifetime of the liver cells [14]. Therefore it is most unlikely that loss of control during carcinogen feeding involves a mutational event.

It has been reported that there is 80–90% inhibition of cholesterol synthesis 24 hr after a single LD_{50} dose of aflatoxin [15]. This inhibition, however, is probably caused by fasting since rats eat very little after such a dose. Our studies, in which increased cholesterol synthesis was observed, were not begun until the rats were regaining weight.

Wogan and Newberne [17] have reported

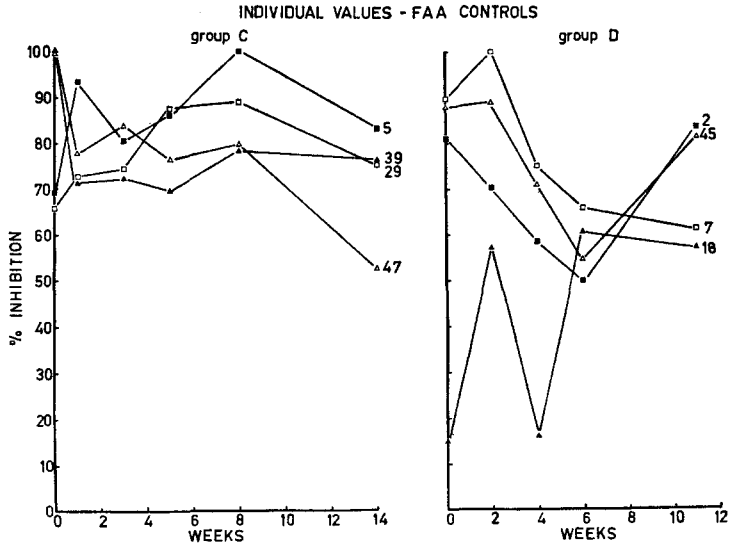

Fig. 4. Individual values for % inhibition of sterol synthesis for the FAA control rats.

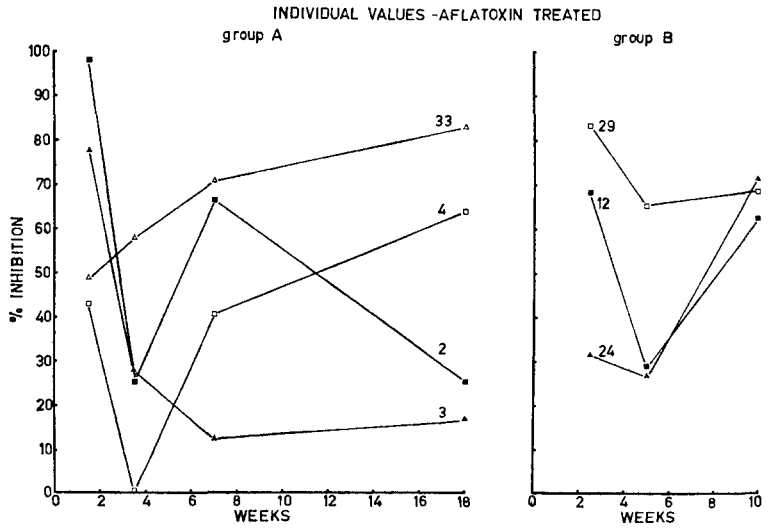

Fig. 5. Individual values for % inhibition of sterol synthesis for several aflatoxin-treated rats.

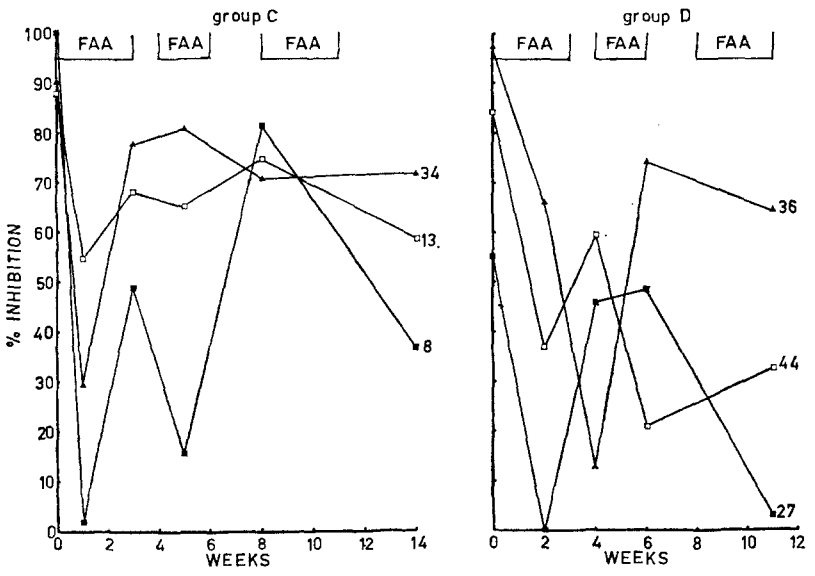

Fig. 6. Individual values for % inhibition of sterol synthesis for several FAA-fed rats.

that after a single LD_{50} dose of aflatoxin all survivors developed histological changes which persisted for as long as 69 weeks, and 1/5 developed hepatomas, with a higher incidence after even longer periods [18]. On the other hand most biochemical changes due to aflatoxin toxicity are gone within a week of a single dose [16], so it is interesting that in our studies the maximum loss of feedback control of cholesterol synthesis was observed 3 to 5 weeks later.

In our present experiments with FAA feeding it is interesting that the animals which developed hyperplastic liver nodules were those which had the greatest loss of control of cholesterol synthesis at 1 and 2 weeks. It has been suggested that hyperplastic liver nodules are part of a sequence between the original liver cell and the hepatic cancer cells [13, 19]. This suggestion is supported by the report of Teebor and Becker [20] that after FAA feeding, in a regimen similar to ours, hepatomas never developed without the prior appearance of nodules, although not all livers which developed nodules did eventually develop hepatomas.

Although our studies presented here have been carried out in the whole animal, we can speculate about what may be happening at the cellular level. It seems reasonable to us that as far as each individual cell is concerned, loss of control should be an all-or-nothing phenomenon. Thus partial loss of control would occur if some cells were affected while others remained normal. The percentage inhibition for the liver as a whole would reflect the proportion of unaffected cells.

This loss of control, which probably could occur in every liver cell, is apparently reversible for the majority of cells, although every hepatoma studied has irreversibly lost this control [1]. We therefore suggest that a few affected cells fail to return to normal and it is these cells that may eventually develop into hyperplastic nodules and then into hepatomas. If only a small proportion of cells fail to revert to normal, then for any given liver the greater the number of cells affected in the first place, the greater the chance that a cell of that particular liver will be permanently altered and thus the greater the probability of hepatoma development.

According to this hypothesis, loss of control would have to occur in a given cell before that particular cell could develop into a tumour, though it is not essential for all cells to be affected. Conversely it would be possible for an animal to show complete loss of control, but never develop a hepatoma, if the change did not become permanent in any individual cell. The probability that any given animal would develop a hepatoma, however, would be related to the degree of early loss of feedback control. We do not suggest that loss of feedback control of cholesterol synthesis actually causes malignancy, only that at present it invariably appears as a property of all malignant and premalignant liver cells.

The vival assay used in these present studies is proving to be extremely useful in our studies of hepatocarcinogenesis, since it gives an indication of which animals have been most affected by a carcinogen at any given time, and the assay can be carried out repeatedly on the same animal. Feedback control of cholesterogenesis, measured by two independent assay systems (vival and vitral) has now been shown to be defective after treatment with two different

carcinogens, thus strongly supporting the hypothesis that the loss of control known in all fully-developed hepatomas is important to the carcinogenic process [3, 5].

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Lower Respiratory Tract Tumours in Syrian Golden Hamsters after Intratracheal Instillations of Diethylnitrosamine alone and with Ferric Oxide*

V. J. FERON†, P. EMMELOT‡ and T. VOSSENAAR§

†*Central Institute for Nutrition and Food Research TNO, Zeist,* ‡*Antoni van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam and* §*Department of Pathology, State University of Utrecht, Utrecht, The Netherlands*

Abstract—*A total of* 37 *tumours of the lower respiratory tract (bronchi, bronchioli and alveoli) was observed in* 62 *male and female Syrian golden hamsters following* 15 *weekly intratracheal instillations of diethylnitrosamine (DENA) alone. The number of tumours in this segment of the respiratory system was* 3 *times higher when ferric oxide was administered simultaneously with DENA* (107 *neoplasms in a total of* 62 *animals examined). The neoplastic response of the lower respiratory tract mainly consisted of adenomas in the bronchiolo-alveolar region, although bronchial papillomas and a few peripheral carcinomas were also observed.*

The well-known, marked carcinogenic effect of DENA on the upper respiratory tract of the Syrian golden hamster was confirmed.

INTRODUCTION

IN SYRIAN golden hamsters the respiratory tract is the main target organ for the carcinogenic action of diethylnitrosamine (DENA) irrespective of the route of administration [1–8]. The tumour response to DENA, however, appears to vary considerably in the different segments of the respiratory tract. In general, the broncho-alveolar system of the hamster is less susceptible to the carcinogenic action of DENA than the upper respiratory tract is [3–5, 7–9].

Ferric oxide (haematite) has been extensively tested for possible carcinogenic activity, but always with negative results [9–11]. In experimental respiratory carcinogenesis ferric oxide has been introduced as a carrier for particles of carcinogenic chemicals. Intratracheal instillation of such mixtures appeared an effective method for the induction of respiratory tumours in hamsters [11]. The essential property of the ferric oxide particles has been considered their capacity to facilitate the penetration and retention of the carcinogen in the lung tissue. However, this explanation does not cover observations of Montesano *et al.* [7, 9, 12] that subcutaneous treatment of hamsters with DENA, followed by intratracheal instillation of ferric oxide resulted in a much higher incidence of broncho-alveolar tumours than DENA treatment alone did. Moreover, in a recent report Harris *et al.* [13] mentioned that ferric oxide may act as a co-factor in respiratory carcinogenesis, because they observed a marked basal cell hyperplasia of the tracheobronchial epithelium in hamsters when multiple doses of ferric oxide were administered intratracheally.

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In the present experiment the role of ferric oxide in pulmonary carcinogenesis was investigated by comparing the effect of intratracheal instillation of ferric oxide and DENA, either alone or in combination.

MATERIAL AND METHODS

Materials

Diethylnitrosamine (DENA) was obtained from EGA-Chemie K.G., Keppler and Reif, Steinheim/Albuch, W. Germany. Its purity was checked by gas–liquid chromatography. Ferric oxide (haematite, particle size distribution: 99% < 5 μm, 98% < 3 μm, 95% < 1 μm) was obtained from Fisher Scientific Co., Fair Lawn, N.J., U.S.A., and the saline solution (0·9% sodium chloride, sterile, non-pyrogenic) from Baxter Laboratories, A. Christiaens N.V., Brussels, Belgium. A 1·5% solution of DENA in saline (v/v) and a 1·5% ferric oxide suspension in saline or in 1·5% DENA (w/v) were prepared and stored at 4°C (maximally 4 weeks) in quantities sufficient for 1 instillation of all hamsters in a certain group.

Animals

Syrian golden hamsters, obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands, were separated by sex and housed in sets of 5 in screen-bottom cages in a temperature-controlled room at 24 to 26°C. The animals were fed a pelleted stock diet (Muracon I, Trouw & Co., N.V., Amsterdam—Putten, The Netherlands) and tap water *ad libitum*. The hamsters were about 11 weeks old when the experiment was started.

Experimental design and conduct

Four groups of hamsters, each consisting of 35 males and 35 females, received a course of 15 weekly intratracheal instillations. For the various groups each instillation consisted of 0·2 ml saline, 3 mg ferric oxide suspended in 0·2 ml saline, 0·2 ml 1·5% DENA in saline or 3 mg ferric oxide suspended in 0·2 ml 1·5% DENA in saline. Before each treatment the animals were lightly anaesthetized with freshly distilled ether. The instillation procedure was not essentially different from the one used by Saffiotti *et al.* [11]. At the end of the treatment period (week 15) 3 males and 3 females out of each group were sacrificed for pathological examination. The experiment was terminated after 78 weeks. Hamsters that died spontaneously or were killed when moribund were autopsied. For killing the animals, and removing and processing the lungs, the technique described in detail by Saffiotti *et al.* [11] was used. Following fixation in 10% neutral formalin, the head (after decalcification of the skull in nitric acid), larynx, trachea, pulmonary lobes, liver, kidneys and other organs showing gross pathology were embedded in paraffin, sectioned (larynx, trachea and pulmonary lobes at 3 levels) and stained with haematoxylin-eosin and other stains when required.

RESULTS

Mortality and body weights

Hamsters treated with DENA or DENA plus ferric oxide showed a much higher mortality and significantly lower body weights than did saline treated controls (Fig. 1). Ferric oxide treatment affected neither survival rates nor body weights. Death in animals given DENA or DENA plus ferric oxide was almost exclusively due to asphyxia resulting from obstruction of the tracheal lumen by papillomas.

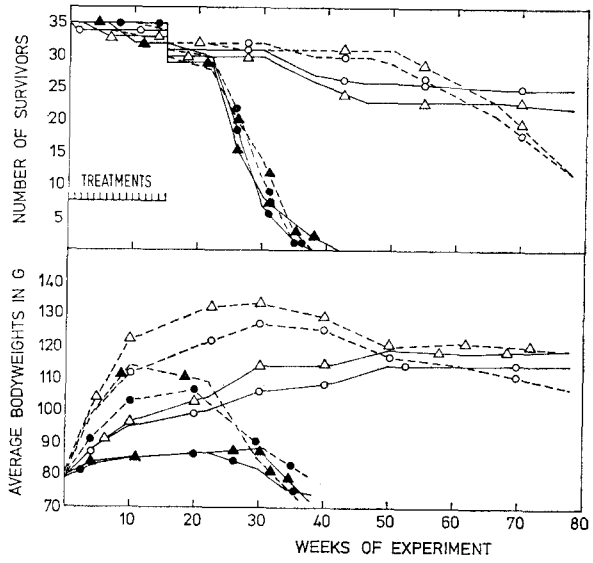

Fig. 1. Survival rates and average body weights of male (——) and female(- - -) Syrian golden hamsters treated intratracheally with saline (△), ferric oxide (○), DENA (▲) or DENA plus ferric oxide (●). At the end of the treatment period 3 males and 3 females of each group were killed for interim information.

Tumours of the lower respiratory tract

Small papillomas of the main-stem bronchi were observed in 4 males treated with DENA alone and in 11 hamsters given DENA plus ferric oxide (Table 1).

Single or multiple adenomas in the bronchiolo-alveolar region (Figs. 2–5) were observed in 21 hamsters treated with DENA alone and in

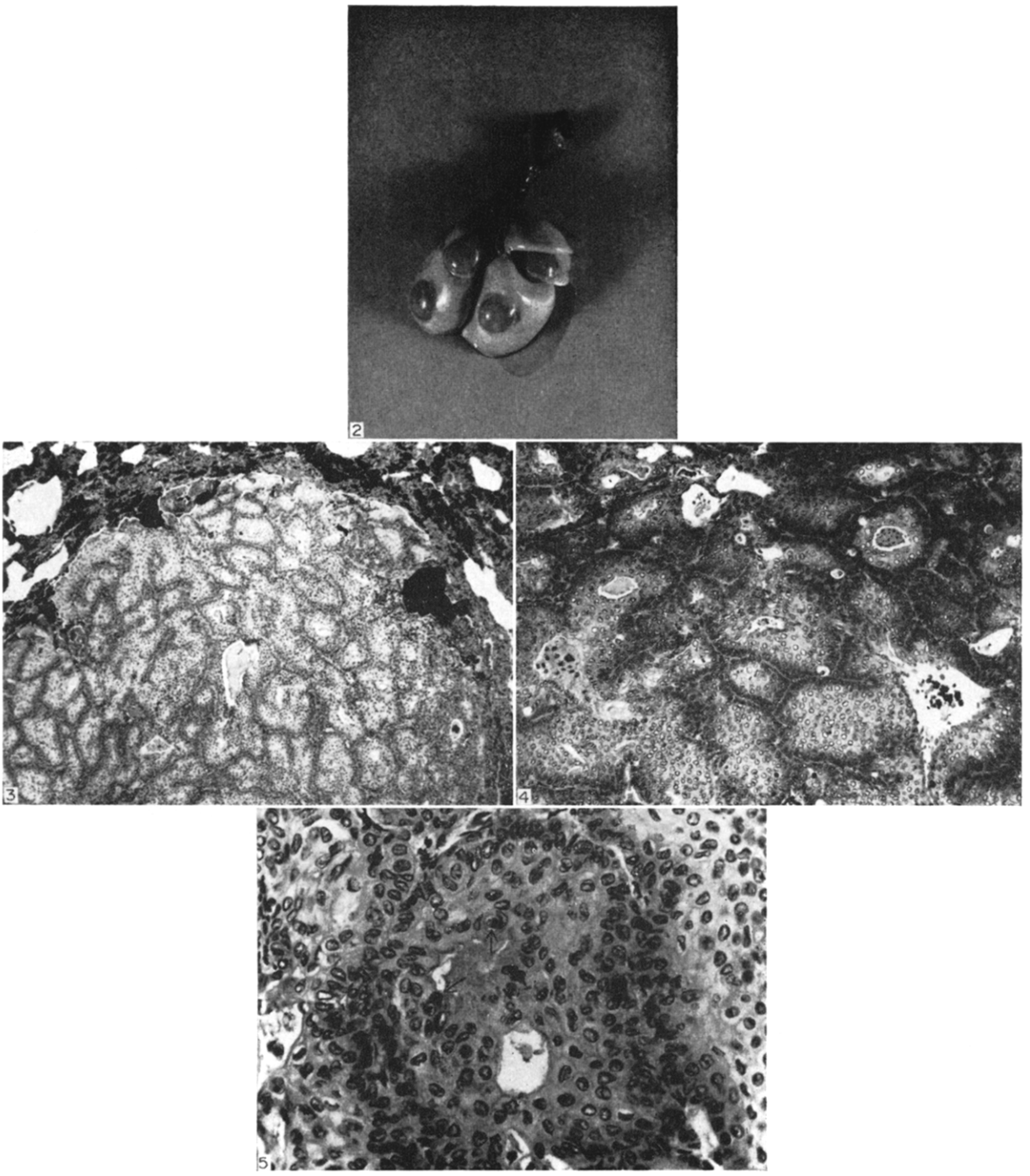

Fig. 2. *DENA-induced peripheral lung tumours.*

Fig. 3. *Peripheral pulmonary adenoma. Adenomatous structures lined by stratified squamous-like epithelium. Note the massive amounts of ferric oxide in the pulmonary tissue adjacent to the tumour* (*DENA plus ferric oxide, Kreyberg's stain,* ×56).

Fig. 4. *Characteristic appearance of DENA-induced pulmonary adenoma* (*DENA plus ferric oxide, Kreyberg's stain* ×140).

Fig. 5. *Pulmonary adenoma. A few mucin-producing cells* (*alcian green positive; arrows*) *are visible* (*DENA, Kreyberg's stain,* ×370).

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Table 1. *Type and incidence of tumours of the lower respiratory tract in Syrian golden hamsters given a course of* 15 *weekly intratracheal instillations of saline, ferric oxide, DENA or DENA plus ferric oxide.*

Site and type of tumour	The number of tumour-bearing animals															
	Animals killed at the end of the treatment period								Animals that died spontaneously or were killed terminally or when moribund							
	Saline		Ferric oxide		DENA		DENA + ferric oxide		Saline		Ferric oxide		DENA		DENA + ferric oxide	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Number of animals examined*	3	3	3	3	2	3	3	3	26	26	29	28	27	30	28	28
Small papilloma of the stem bronchi	0	0	0	0	0	0	0	0	0	0	0	0	4	0	4	7
Bronchiolo-alveolar adenoma:																
One single adenoma	0	0	0	0	0	0	1	1	0	0	0	0	6	7	10	3
Number of multiple adenomas: 2	0	0	0	0	0	0	2	0	0	0	0	0	3	3	2	4
3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2
4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
5–13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1
Small-sized peripheral carcinoma	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
Total number of lower respiratory tract tumours	0	0	0	0	0	0	5	1	0	0	0	0	24	13	67	34

*Initially, each group consisted of 35 male and 35 female hamsters.

33 which had received DENA plus ferric oxide. The total number of bronchiolo-alveolar adenomas in the DENA-group was 32 and that in the DENA plus ferric oxide-group was as high as 94. Of the 6 animals of each group killed at the end of the treatment period, 4 treated with DENA plus ferric oxide had already developed such adenomas, whereas in the animals of the other groups these tumours were not found at all at this time.

Grossly, the adenomas appeared as greyish-white, firm nodules measuring 0·1 to 1 cm in diameter (Fig. 2). Microscopically, they appeared to consist predominantly of acinar structures lined by stratified, squamous-like epithelium (Figs. 3, 4). The cells were round to oval and rather uniform, had distinct cell boundaries and moderate amounts of pale cytoplasm (Figs. 4, 5). Superficial cell layers were often flattened, but keratinization was never seen. Intercellular bridges could not be identified unequivocally. The cytoplasm of an occasional superficial tumour cell contained alcian green positive material, indicating mucin formation (Fig. 5). Several of the larger neoplasms had a wide central lumen, surrounded by clusters of the adenomatous structures described. Columnar epithelium was encountered in a few tumours. Transition from this type of epithelium to stratified epithelium was sometimes visible. The tumours invariably were circumscript.

Three small-sized peripheral tumours, showing characteristics of squamous cell or anaplastic large cell carcinomas, were found among DENA or DENA plus ferric oxide treated animals.

In addition to the tumours in the bronchoalveolar system, marked cellular and nuclear atypism and areas of atypical hyperplasia were noticed in the bronchial lining epithelium of all animals given DENA or DENA plus ferric oxide, but in none of the saline- or ferric oxide-treated hamsters. Degree and frequency of these changes were comparable in both treatment groups.

Peribronchiolar adenomatoid lesions of a minimal extension were seen mostly in ferric oxide-treated animals which survived the experimental period, but occurred also in a few animals of each of the other groups.

A few small patches of alveolar mucous cell metaplasia were found in 1 or 2 hamsters of the various groups, including the saline-treated control group.

Tumours of the upper respiratory tract

Many tumours of the nasal cavity (36%), moderate numbers of laryngeal papillomas

(7%) and a nearly 100% incidence of tracheal papillomas were noticed in hamsters treated with DENA or DENA plus ferric oxide. There was no distinct difference in incidence of these tumours between both groups. The morphology of these upper respiratory tract tumours was the same as described previously [4, 5, 7], except for the presence of squamous epithelium in tracheal papillomas which could not be identified unequivocally in the present material. The latter observation is in agreement with recent ultrastructural work on DENA-induced tracheal papillomas in hamsters, the results of which did not give evidence of metaplastic change to squamous epithelium in these tumours [14, 15].

Other tumours

In the liver 4 hepatocellular carcinomas and one cholangioma were observed in 3 males and 1 female treated with DENA plus ferric oxide. In addition, the following tumours, unrelated to DENA-treatment, were found: 1 thyroid adenoma, 2 adrenal cortical adenomas, 1 sarcomatous tumour and 1 ovarian tumour.

DISCUSSION

The results of this experiment confirm the finding of other investigators that DENA has a marked carcinogenic effect on the upper respiratory tract of the Syrian golden hamster [3–9]. With regard to type and incidence of DENA-induced tumours in the lower respiratory tract of the hamster, some discrepancies are noticeable in the literature as has been discussed in detail by Montesano and Saffiotti [7]. The neoplastic response of the lower respiratory tract to DENA alone, observed in the present study, was less than that obtained by Dontenwill *et al.* [1, 2], but higher than that reported by the other authors [3, 5–8]. Our findings, however, seem to be in keeping with a recent statement by Montesano *et al.* [9] that Dontenwill had repeated his previous experiments with DENA in hamsters [1, 2] and now observed much lower incidences of tumours of the broncho-alveolar system. Moreover, when Montesano *et al.* [9] administered the maximal tolerated dose of DENA to adult hamsters, they found a higher incidence of tumours in the lower respiratory tract than in their former experiments [9].

The neoplastic response of the lower respiratory tract in our hamsters mainly consisted of benign tumours considered to be derived from the bronchiolo-alveolar system and showing adenomatous structures predominantly lined by stratified epithelium. The histological appearance of these tumours resembles that of the benign peripheral adeno-squamous neoplasms or bronchiolar "papillomas" reported previously in hamsters after treatment with various carcinogens [9, 10, 12].

In comparison with the results of DENA-treatment alone, those obtained after the concurrent intratracheal instillation of DENA and ferric oxide were indicative of a shortening of the latent period of bronchiolo-alveolar adenomas and showed a marked increase in the tumour yield of the lower respiratory tract. This finding is in keeping with the observation of Montesano *et al.* [9] that subcutaneous treatment of Syrian golden hamsters with DENA followed, 5 weeks later, by intratracheal instillation of ferric oxide resulted in a much higher incidence of broncho-alveolar tumours than DENA-treatment alone did. Ferric oxide, therefore, might be considered a cocarcinogen.

As tracheal papillomas in DENA-treated hamsters developed relatively early, the high incidence of pulmonary tumours in these animals could be due to transplantation of the tracheal papillomas into the lower respiratory tract as a consequence of the instillation procedure. The presence of ferric oxide particles may facilitate the transplantation and explain the increased incidence of lung tumours in animals treated with DENA plus ferric oxide. In this connection the morphology of the lung tumours is very interesting and, therefore, electron microscopical studies on this type of DENA-induced tumours have meanwhile been undertaken. The results obtained so far, are indicative of alveolar cell origin of these pulmonary neoplasms [16].

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Agglutination of Leukemic Myeloblasts by Neuraminidase

CHR. SAUTER*, J. LINDENMANN† and A. GERBER*

**Division of Oncology, Department of Medicine, and †Division of Experimental Microbiology, Institute of Medical Microbiology, University of Zürich, 8006 Zürich, Switzerland*

Abstract—*Peripheral white blood cells from seven patients with acute myeloblastic leukemia were isolated and then incubated with various concentrations of vibrio cholerae neuraminidase. Agglutination of leukemic myeloblasts was observed. This agglutination occurred after about two hours of incubation if the medium contained 20 U/ml of neuraminidase. With 0·2 U/ml of the enzyme agglutination was observed only after 24 hr of incubation. The determination of the exact mechanism of this agglutination will be of great interest since tumor specific antigens could be involved.*

INTRODUCTION

THE TREATMENT of different cells by neuraminidase results in a change of many surface properties: Reduction of electrophoretic mobility [1], increased immunogenicity [2, 3], decreased reaggregation [4], reduced adhesion to glass [5], enhanced phagocytosis [6] and destruction of receptors for myxoviruses [7] and polyoma viruses [8]. In this report we describe another effect of neuraminidase, which is probably also due to cell surface changes.

During experiments with myxoviruses and peripheral white blood cells from patients with acute myeloblastic leukemia (AML) we noted regularly an agglutination of leukemic cells starting after about 10 hr of incubation. In control cultures this agglutination was not observed or was much less pronounced. When the viruses were inactivated by u.v. irradiation, the agglutination still occurred. This suggested that the agglutination could be due to the neuraminidase content of the virus. This observation prompted us to initiate experiments with Vibrio cholerae neuraminidase and peripheral white blood cells from patients suffering from AML.

Accepted 9 March 1972.

MATERIAL AND METHODS

The isolation of peripheral white blood cells from seven patients suffering from AML was done according to the procedure adopted in this laboratory [9]. After washing the cells were resuspended at a concentration of 5×10^6 cells per ml in minimum essential medium on Earle's base supplemented with 15% fetal calf serum, penicillin (400 IU/ml) and streptomycin (400 μg/ml). To aliquots of the cell suspension neuraminidase was added so that the following final concentrations of neuraminidase were obtained: 20 U/ml, 2 U/ml and 0·2 U/ml. The undiluted neuraminidase solution had a quoted strength of 500 U/ml, (Behringwerke, batch No. 869 AR, source Vibrio cholerae culture; one unit is the amount of the enzyme to release 1 μg of N-acetylneuraminic acid from human α-acid glycoprotein in a sodium acetate buffer solution 0·05 mole/l, pH 5·5, with an addition of 9 mg of sodium chloride and 1 mg of calcium chloride per ml in 15 min at 37°C). 2 ml of control and neuraminidase containing cell suspensions were incubated at 37°C in plastic Petri dishes (Falcon Plastics, diameter 35 mm).

The pH was kept stable in an incubator with constant pCO_2. The cells were observed by phase contrast and dark ground microscopy.

Dry smears were made at 0, 2, 10, 24 and 55 hr and stained with Giemsa. Cell viability was assessed by exclusion of trypan blue and was always better than 90% up to 24 hr of incubation.

RESULTS

In neuraminidase-containing cultures an agglutination of myeloblasts was observed (Fig. 1a and 1b), whereas in control cultures agglutination did not occur or was much less pronounced (Fig. 1c and 1d). By Giemsa staining we found the cell clusters to consist of myeloblasts. The beginning of the agglutination depended on the concentration of neuraminidase in the medium (Table 1).

Table 1. *Agglutination of leukemic myeloblasts with vibrio cholerae neuraminidase*

Neuraminidase concentration		Hours of incubation				
		0	2	10	24	55
20	U/ml	−	+	++	++	++
2	U/ml	−	−	+	++	++
0·2	U/ml	−	−	−	+	++
0		−	−	−	−	−

− no or very little agglutination
\+ clear agglutination
++ heavy agglutination

DISCUSSION

The mechanism of the agglutination of leukemic myeloblasts by neuraminidase is unknown. There are at least three possible explanations: (*a*) The action of neuraminidase is to liberate sialic acid moieties from cell surfaces [10]. Leukemic myeloblasts, after loosing sialic acid from their surfaces, could behave like more differentiated cells from the granulocytic series which are known to clump spontaneously *in vitro*. This could be due to changes in electric surface charges [1].

(*b*) It has been shown for several human tumors that the patient's peripheral blood lymphocytes are able to destroy the same patient's tumor cells *in vitro*, provided no blocking factors from the patient's serum are added [11]. It is also known that one can increase the immunogenicity of animal tumor cells by treating them with neuraminidase, probably by exposing tumor-specific surface antigens [2, 3]. In our experiments we are certainly dealing with a mixture of leukemic myeloblasts and normal peripheral blood lymphocytes. One can argue that the neuraminidase treatment exposes also in this system tumor-specific antigens on the cell surface. As the first step of a cytotoxic action the already sensitized lymphocytes would then make the tumor cells agglutinate. (*c*) The action of the neuraminidase could be on the lymphocytes rather than on the myeloblasts. It has been shown that neuraminidase treatment of spleen cells enhances the immunolysis of target cells [12].

It will be of interest to determine the exact mechanism of this agglutination. Should tumor-specific antigens be involved this type of experiment could offer useful approaches to their study.

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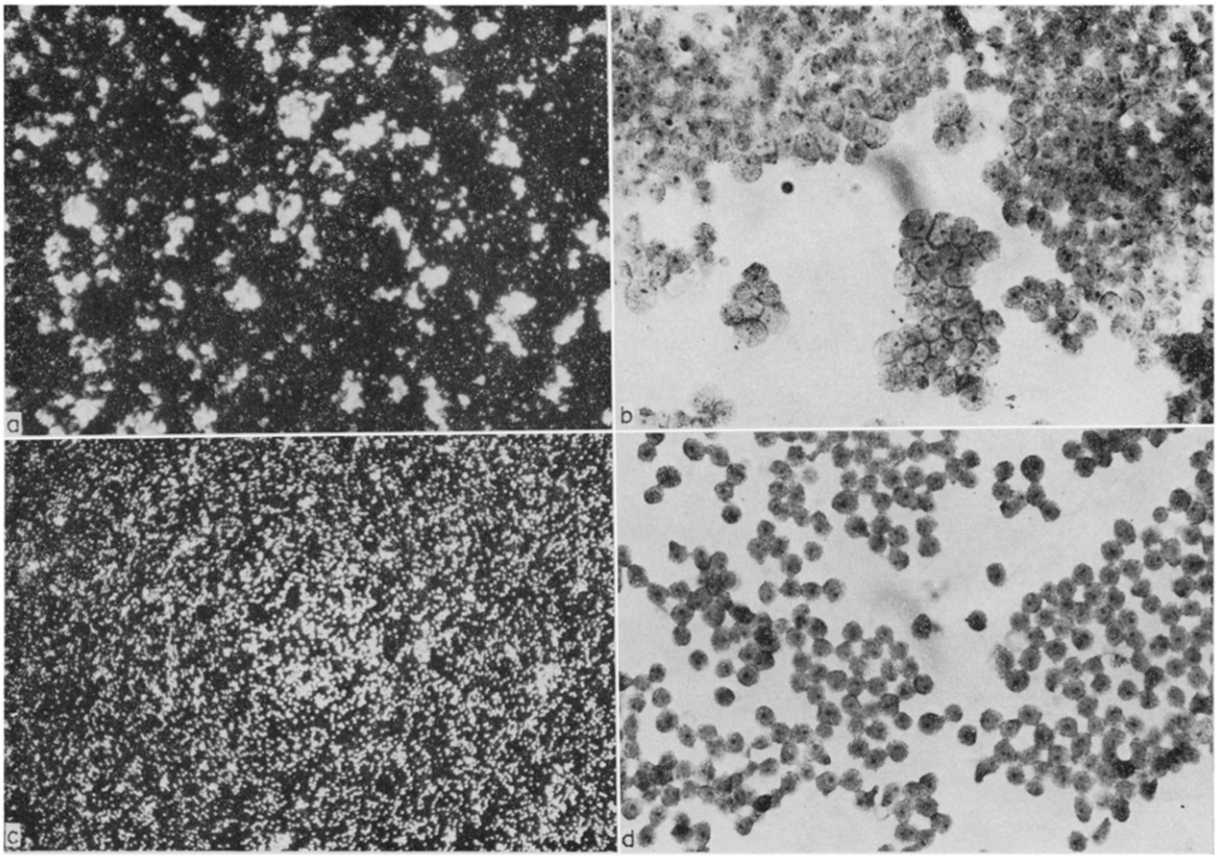

Fig 1. *Cultures of peripheral white blood cells from a patient suffering from acute myeloblastic leukemia,* 24 *hr of incubation. a and b with* 2 *U/ml of neuraminidase, c and d without neuraminidase. a and c dark ground photography of the cell cultures (approx.* × 48). *b and d Giemsa stained smears (approx.* × *480). Note the agglutination of myeloblasts in a and b.*

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Plasma and Urinary Levels of Adriamycin in Man

R. ROSSO*, C. RAVAZZONI†, M. ESPOSITO*, R. SALA* and L. SANTI*

**Cattedra di Oncologia Sperimentale, Università di Genova e Ospedali Civili di Genova, Servizio di Oncologia, viale Benedetto XV. 16132 Genova, Italia. †Ospedali Civili di Genova, I Divisione Pneumologica*

Abstract—*Adriamycin, given to 10 cancer patients at the dosage of 0·4 mg/kg intravenously for three following days (this course of therapy was repeated three times at 10-day intervals), was evaluated in plasma and urine according to a simpler and sensitive fluorimetric procedure. By considering plasma disappearance curves of single patients following the first intravenous injection, quite important differences emerged concerning duration of measurable amounts of the drug. Moreover, adriamycin concentrations in plasma appeared to be progressively more sustained and sometimes more lasting following the subsequent drug injections.*

Urinary excretion of adriamycin turned out to be of a great variability concerning duration of excretion in different patients, duration of measurable excretion in the same patient relative to the successive therapeutic courses and percentage of the total dose administered. Thin-layer chromatography of plasma and urine extracts did not reveal any fluorescent band other than adriamycin itself.

INTRODUCTION

ADRIAMYCIN, 14-hydroxy-daunomycin, has been evaluated for anticancer activity by many clinical groups [1–3]. While a good deal of data about pharmacokinetics [4] and metabolism [5–6] of daunomycin in man is known, a single preliminary study on clinical pharmacology of adriamycin has been reported by Di Fronzo [7] using the isotopically labelled compound.

In this report we have described the same aspects of the clinical pharmacology of adriamycin in man, with particular reference to plasma and urinary levels, evaluated by the fluorescence characteristics of the molecule.

MATERIAL AND METHODS

Subjects

Patients of both sexes participating in the studies had solid unresectable cancer, and except for one patient (R.A.) all had normal renal function. The diagnoses were 7 lung carcinomas, 1 osteosarcoma, 1 renal sarcoma with multiple cutaneous, hepatic and pulmonary metastases, 1 myxo-fibrosarcoma.

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Administration

Adriamycin, kindly supplied by Farmitalia (Milan), was administered at a dose of 0·4 mg/kg for three days; this course was usually repeated three times at ten-day intervals. The administration was performed intravenously by rapid injection, followed by a washing of the vein by saline (250 ml). Blood samples for adriamycin determinations were obtained from the cubital vein contralateral to that used for drug injection 15 min before the first drug administration and after 0·25, 0·50, 2, 4, 8, 12, 24 hr. Total urines were collected at 12-hr intervals until drug was no longer measurable. Administration of any other drug was carefully limited during the three days preceding adriamycin treatment. No blood transfusions were allowed during the whole cycle of treatment.

Fluorescence assay

The determinations of adriamycin levels in plasma, blood cellular fraction and urine were performed according to the procedure described by Finkel [8] for daunomycin determination in biologic fluids. A mixture of 0·5 ml of the biological sample and 0·5 ml of water was extracted by stirring with 2·0 ml of *n*-butyl alcohol and by centrifugation at 4000 rev/min for 15 min. The fluorescence of the supernatant solution was determined in a CGA spectrophotofluorimeter. An activation wavelength of 465 nm and an emission wavelength of 585 nm were used. After the fluorescence of the samples had been measured, fluorescence spectra were determined in order to check quantitative data.

Thin-layer chromatography

Plasma and urine were extracted with *n*-butyl alcohol as above mentioned and chromatographed on 250 μ silica gel H with chloroform: methanol: acetic acid (80 : 20 : 4) or *n*-butyl alcohol: acetic acid: water (4 : 1 : 5). Purified adriamycin and adriamycinone were used as standards.

RESULTS

Recovery studies

After addition of adriamycin standards, extraction with *n*-butyl alcohol allowed determinations of 0·01 μg/ml of plasma and 0·05 μg/ml of urine by the fluorescence assay.

Fluorescence studies

The mean plasma disappearance curve of adriamycin is represented in Fig. 1. Clearance of the drug from plasma was very rapid up to 8 to 12 hr after the intravenous injection; however, by the 24th hour, plasma levels tended to increase slightly. By considering plasma disappearance curves of single patients, quite important differences emerged concerning duration of measurable amounts of the drug. 24 hr after drug administration, adriamycin was not detectable in plasma of several patients while in some other patients a marked increase relative to previous determinations was observed. This pattern of clearance possibly does not allow a correct calculation of half-life.

Plasma levels during the first three-day course of therapy in five patients are represented in Fig. 2. When treatment was performed on subsequent days, adriamycin concentrations in plasma appeared to be pro-

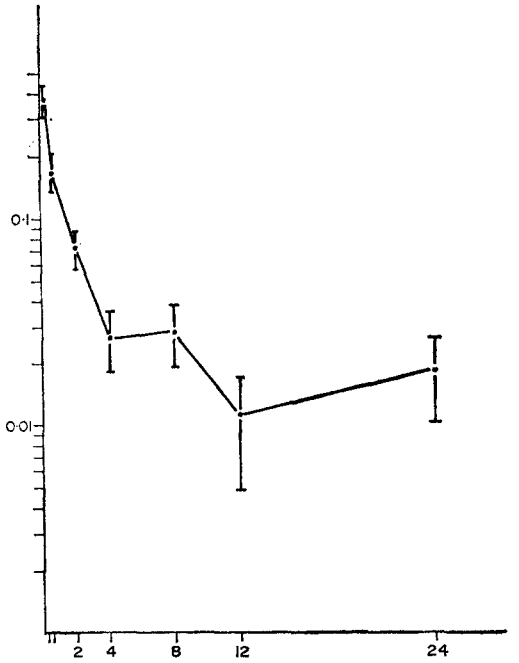

Fig. 1. Plasma adriamycin concentration and standard error of the mean after a single intravenous injection (0·4 mg/kg) *in 7 patients.*
Abscissa: hours after intravenous drug administration.
Ordinate: micrograms/ml of plasma.

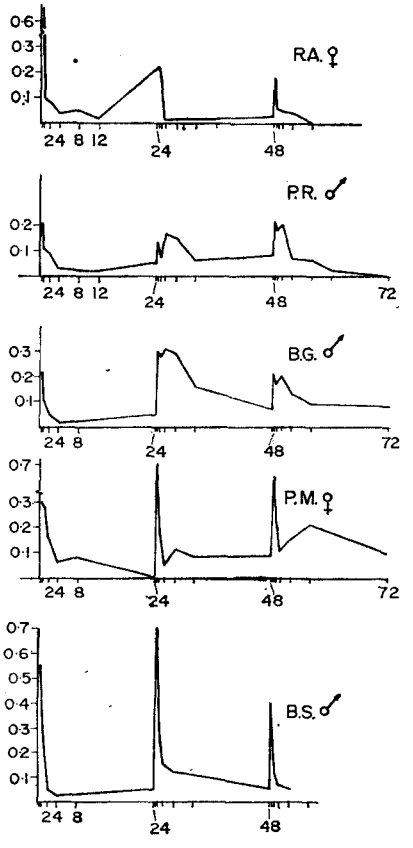

Fig. 2. Plasma levels during the first three-days course of therapy in 5 patients, after intravenous drug administration (0·4 mg/kg).
Abscissa: hours after intravenous drug administration.
Ordinate: drug levels expressed as micrograms/ml of plasma.

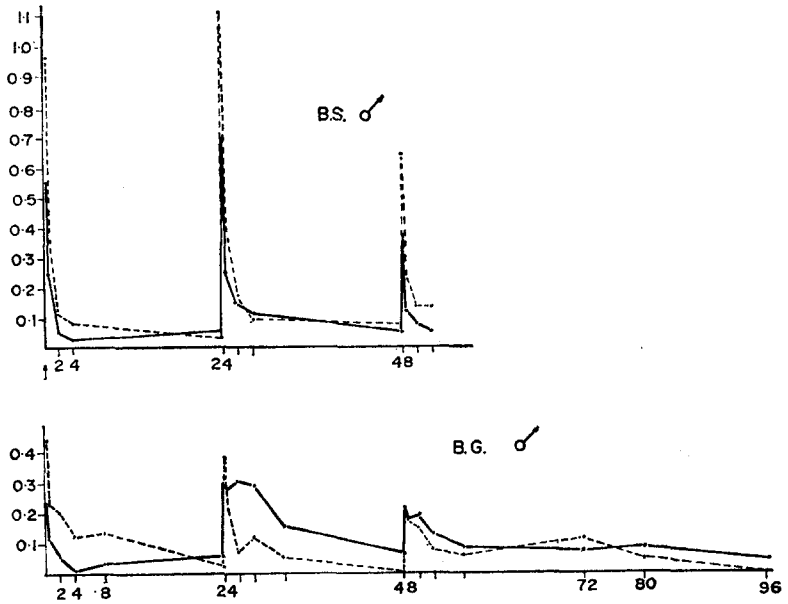

Fig. 3. Adriamycin levels in the blood cellular fraction and in plasma during the first three-days course of therapy in two patients, after intravenous drug administration (0·4 mg/kg).
Abscissa: hours after intravenous drug administration.
Ordinate: drug levels expressed as micrograms/ml.
——— *plasma levels.*
– – – – *blood cellular fraction levels.*

gressively more sustained and sometimes more lasting.

Adriamycin levels in the blood cellular fraction were found to be higher than the corresponding plasma levels up to 8 hr after the first adriamycin intravenous administration (Fig. 3). These data probably account for a storage of the drug in/on the blood cells; however, this storage did not occur when treatment was repeated on subsequent days.

Urinary excretion of adriamycin (Fig. 4) turned out to be of a great variability concerning duration of measurable excretion in different patients, duration of measurable excretion in the same patient relative to the successive therapeutic courses and percentage of the total dose administered. In fact considering the first three-day course of therapy, the duration of excretion ranged from a minimum of 72 hr in a patient with impaired renal function and of 120 hr in a patient with normal renal function, to a maximum of 216 hr. Moreover, in all the patients but the one with impaired renal function, duration of urinary excretion of adriamycin increased from the first three-day course to the subsequent therapeutic courses: from 216 to 324 hr in patient P.M., from 120 to 144 and then to 240 hr in patient P.R., from 144 to 204 hr in patient BG.

Excretion expressed as a percentage of the total dose administered was very variable among patients. Relative to the first three-day course, the percentages of the total urinary excretion of the drug ranged from 9·6 in the patient with impaired renal function and 28·0 in a patient with normal renal function to 51·1%. Determination of adriamycin in faeces has been attempted. The presence of interfering fluorescent materials did not allow a satisfactory measurement.

Chromatographic studies

Thin-layer chromatography performed on *n*-butyl alcohol extracts of plasma and urine samples obtained at the same time intervals used for the fluorescence studies did not reveal in any case fluorescent bands other than that of adriamycin itself.

DISCUSSION

Preliminary studies in mice by Di Fronzo and co-workers using the isotopically labelled adriamycin [9] and by us using fluorescence analysis [10] showed a rapid clearance of the drug plasma and a marked uptake in tissues, particularly in spleen, lymph nodes and bone marrow. This pattern of distribution was similar to that of daunomycin, except for lymphatic tissue where adriamycin was much longer lasting than daunomycin. In fact very high levels were measurable in spleen up to 96 hr after a single intravenous injection of 5 mg/Kg of adriamycin.

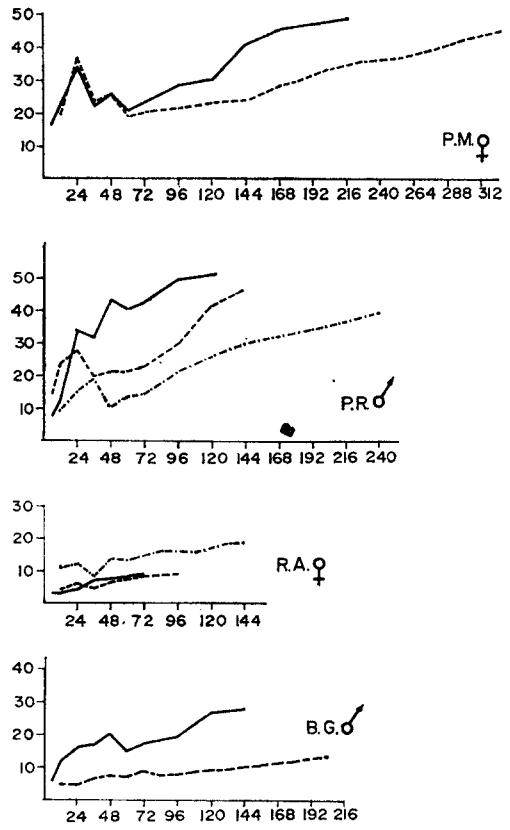

Fig. 4. Urinary excretion of Adriamycin (0·4 mg/kg intravenously) administered on three subsequent days to 4 patients. Excretion is expressed as per cent of total dose administered at each time. Determinations were performed until drug was no longer measurable.
Abscissa: time intervals of determinations.
Ordinate: percentage of total dose of adriamycin.
——— *1st cycle*
– – – – *2nd cycle*
– · – · – *3rd cycle*

On the basis of data by Di Fronzo and coworkers using tritiated adriamycin [4] and the results here reported by us following the fluorescence characteristics of the molecule adriamycin very probably behaves in a similar way in man. However, several differences must be pointed out between our data and those by Di Fronzo. In fact in our data clearance of adriamycin from plasma is very rapid and in several patients no drug was measurable 24 hr after the intravenous injection. According to Di Fronzo, adriamycin levels were similar up to 260 hr after the intravenous administration to levels observed as soon as after 8 to 12 hr. It is difficult to define whether the quite longer plasma levels of adriamycin reported by Di Fronzo are due to relevant pharmacokinetic differences of his patients or to the fact that tritiated chemicals other than adriamycin have been measured.

The similarity of the disappearance curves reported by Di Fronzo and by us up to 24 hr after the drug administration reveals that differences are not due to sensitivity of the procedures employed.

When treatment is repeated on subsequent days, clearance of adriamycin from plasma is slower and plasma levels tend to become progressively higher and more lasting. These plasma levels probably account for an accumulation of drug in tissues. From data in animals, tissue accumulation seems to be relevant even after a single administration. On this basis further investigation will have to be paid to the importance of treatment schedule—intermittent or on subsequent days—in relation to the therapeutic and toxic effects.

A peculiar feature is represented by the higher level of adriamycin in the corpuscular fraction than in corresponding plasma up to 8 hr after the first intravenous injection. Since this time both plasma and corpuscular fraction levels tend to overlap. It will be of interest to determine whether drug is preferentially accumulated in red or white cells; unpublished data on daunomycin show a preferential accumulation in leucocytes (three to five hundred times more than in red cells). Urinary excretion is quite important in the total excretion of the drug: probably faecal excretion is equally important because of the high excretion with bile [11]. The long lasting urinary excretion accounts for the long lasting persistence of adriamycin in tissues and can be kept in mind in order to achieve a cytotoxic activity at the urinary level.

Chromatographic studies did not provide any evidence of metabolites of adriamycin in plasma and urine; these data agree with similar unpublished data from other authors. Moreover the absence of metabolites of adriamycin in biological fluids means that fluorescence measurements are due only to adriamycin itself. This is not true for daunomycin, however, because several of its metabolites have the same fluorescence characteristics as the parent compound. In this case fluorescence measurements can be due to daunomycin as well as to several of its metabolites; only chromatographic separation can be distinctive.

Concluding, we like to stress two points. First of all, the apparent total lack of metabolism of adriamycin while for the analogous daunomycin several metabolites are well known [6–7]. Secondarily, the method here described for adriamycin is very rapid and sensitive and it can be employed for routine evaluation of adriamycin levels in order to make correlations between pharmacokinetic data and biological effect.

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Biochemical, Histochemical and Ultrastructural Studies of Changes Occurring with Growth of Ehrlich Ascites Cells *in vivo*

P. A. FLETCHER

Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds 2, Great Britain

Abstract—*During growth of an Ehrlich ascites tumour* in vivo *there was a fall in TdR-kinase specific activity which was not simply related to the concurrently lowered* ^{3}*H-TdR labelling index. Accompanying this fall, there was a decrease in histochemically-detectable glucose-6-phosphate dehydrogenase (G6PDH) activity, which could be reversed by reduction of tumour mass by aspiration or, more transiently by intraperitoneal injection of pyruvate. Histochemically-detectable succinic and lactic dehydrogenase activity remained unchanged with tumour growth. Biochemical assays of G6PDH activity, indicated a constant specific activity accompanying tumour growth.*

The results of ultrastructural studies of tumour cells 5 *and* 15 *days after transplantation showed an increased cellular lipid content for the older tumour, together with mitochondrial abnormalities and decreased cytoplasmic density.*

The results suggested that, during the growth of this tumour in vivo, *there is a decrease in proliferative activity accompanying a general slowing of metabolic processes with decreased oxygen availability as an important causative factor.*

INTRODUCTION

DURING the evolution of several forms of ascitic tumour grown in mice, there is an alteration of growth kinetics [1–4]. This alteration is characterized by a progressive diminution in the proportion of cells synthesizing DNA, a prolongation of cell cycle time and a fall in growth fraction. Among the changes noted in a previous study [5] of an Ehrlich ascitic tumour in this laboratory, were an increase in modal cell size and an accumulation of cells with post-mitotic, pre-DNA-synthetic, DNA contents, which were not labelled after multiple injections of tritiated thymidine but retained the ability to divide after reduction of the tumour mass.

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Harris *et al.* [6] concluded that availability of oxygen was probably an important factor in the control of cell division in ascites tumour cells *in vivo*. The present study reports further investigations of metabolic changes in Ehrlich ascites cells in log and lag phases of growth, particularly the activity of thymidine kinase, and glucose-6-phosphate dehydrogenase.

MATERIALS AND METHODS

The techniques for transplantation of Ehrlich ascites cells (5×10^6) into male A mice, and for cell counting and volume measurement using a Coulter Counter, have been described previously [5].

Radiochemicals

Tritiated thymidine (^{3}H-TdR), (specific activity 5Ci/mM), ^{14}C–TdR (specific activity

60 mCi/mM), and thymidine 2-C14-5′ monophosphate (specific activity 57 mCi/mM) were obtained from the Radiochemical Centre, Amersham, England.

^{3}H-TdR labelling indices

The determination of labelling indices was made by incubating 0·1 ml of ascites tumour fluid ($1–10 \times 10^6$ tumour cells) for 20 min in Hanks' medium containing ^{3}H-TdR at 5μCi/ml. Subsequently the cells were washed, smeared on glass slides, fixed in absolute methanol for 10 min and treated for autoradiography as previously described [5]. Two thousand cells were counted for the determination of the percentage of cells labelled.

Measurement of enzyme activities

Histochemical methods

Succinic (*EC* 1.3.99.1), *lactic* (*EC* 1.1.1.27) *and glucose-6-phosphate* (*EC* 1.1.1.49) *dehydrogenases.* (*SDH, LDH and G6PDH respectively*). Activities were estimated in air-dried, unfixed smears of unwashed tumour cells by the method of Hayhoe *et al.* [7]. The smears were ringed with vaseline and incubated under coverslips at 37°C for 60 min for SDH, 30 min for G6PDH and 10 min for LDH detection. On completion of staining the smears were washed briefly in distilled water, air-dried, the vaseline removed and the preparations finally mounted in Zeiss immersion oil (R_f (20°C) 1·515). Three smears were treated for any one tumour sample and run in conjunction with negative (no substrate or coenzyme) and maximally positive controls (tumour cells 6 days following transplantation). Staining intensities were classified into 4 grades, grade 1 being of lowest intensity. The latter cells were too pale to photograph adequately. Figures 2, 3 and 4 illustrate grades 2 to 4 and represent smears stained simultaneously with the same incubation mixture and photographed under exactly similar conditions.

Biochemical methods

Thymidine kinase (*TdR-kinase*). Tumour cells were washed with 0·9% NaCl in deionised water and subsequently treated at a concentration of 5×10^6 tumour cells per ml, as described by Bianchi *et al.* [8]. For the kinase assay, 0·2 ml aliquots of the supernatant fraction were added to 0·8 ml of incubation medium containing 5·6 μmole of 3-phosphoglyceric acid, 6·8 μmole of Mg^{2+}, 6·6 μmole ATP, 10 mμmole ^{14}C-TdR or ^{3}H-TdR (or ^{14}C-TMP), 616 μmole KCl and 50 μmole Tris HCl. This mixture was incubated aerobically with shaking, for 15, 30 and 45 min at 37°C and the reaction terminated by cooling and adding 0·1 ml 100% (w/v) trichloroacetic acid. Reaction rates were always linear under these conditions. The TCA insoluble precipitate was removed by centrifugation and 20 μl of the acid soluble supernatant analysed by descending chromatography on Whatman No. 1 paper with *n*-butanol:acetone:glacial acetic acid:water:ammonia (14:10:6:9:1) as a solvent [9].

The distribution of ^{14}C radioactivity on the chromatograms was measured with an Actigraph III chromatogram scanner (Nuclear Chicago). When ^{3}H-TdR was used (the first tumour pair of Table 1) the chromatograms were cut into multiple segments and the pieces of paper counted in a Packard Tricarb Scintillation Spectrometer, using scintillation fluid composed of 5 g 2,5 diphenyl oxazole, and 0·3 g 1, 4, Bis 2 (4 methyl 5 phenyl oxazolyl)-benzene per litre of toluene. The counts were made at gain and window settings of 55% and 150–1000 respectively.

At the cell concentration used, the activities of other kinases catalysing the formation of TDP or TTP were inappreciable as activity was not detectable in the regions of the chromatogram corresponding to these latter two compounds.

The degradation of thymidine (investigated by using the normal kinase incubation mixture with TdR but without ATP) by the tumour extracts did not exceed control activities. TMP degradation (investigated using ^{14}C-TMP in the incubation mixture in place of ^{14}C-TdR), although marginally higher in the 15-day than in the 6-day tumour tested, was so low (0·036% TMP-degraded/min/10^6 tumour cells) that it could be neglected.

Glucose-6-phosphate dehydrogenase. The cells were washed, disrupted [10] and G6PDH activity assayed according to the method of Glock and McLean [11]. Smears were made to check that all cells had been disrupted for the two tumour ages compared. The assay methods used were based on two theories: (1) ignoring any 6-phosphogluconic acid dehydrogenase (6PGDH) present and using excess of glucose-6-phosphate (G6P) as substrate (this corresponds to the theoretical basis for the histochemical method); (2) making allowance for 6PGDH activity. The summation of 6PGDH and G6PDH activities is determined using excess 6-phosphogluconic acid (6PGA) + G6P. The activity of 6PGDH alone is assessed using excess 6PGA as substrate. The difference between the two assessments is equivalent to any G6PDH activity.

Histochemical stains

Lipid stains

Sudan Black B. Smears were fixed in formol calcium for 18 hr, rinsed in distilled water, stained in saturated Sudan Black B in 70% ethanol for 30 min, rinsed briefly in 50% ethanol, dried by inverting on to filter paper and gently pressing and mounted in glycerin jelly.

Oil Red O. (Neutral fats.) According to Pearse [12].

Negative control for lipid stains. Cells extracted with methanol:chloroform (3:1) for 48 hr prior to smearing.

The Periodic Acid Schiff Reaction (PAS). According to Lillie [13].

Acid phosphatase (after Gomori). Smears were fixed for 5 min in absolute acetone at 4°C and subsequently incubated for 30 min at 37°C in the following medium: 0·05 M acetate buffer pH 5·0, containing 0·0033 M lead nitrate, 0·0087 M sodium-β-glycerophosphate, pre-incubated at 37°C for 1 hr and filtered just prior to use. The slides were then briefly rinsed in distilled water, 1% acetic acid and distilled water again. They were then immersed for 2 min in 1% ammonium sulphide in distilled water, briefly rinsed in distilled water, counter-stained with Harris' haematoxylin, rinsed in tap water, air-dried and mounted in Zeiss immersion oil.

Viability studies. Viable and non-viable ascites cells were distinguished by staining with Nigrosin [14].

Electron microscopy. The tumour cells were washed with Hanks' medium, fixed as a pellet in paraformaldehydeglutaraldehyde [15] and subsequently treated and stained as described elsewhere [16]. The method of Seligman *et al.* [17] was used to enhance the contrast of lipid-containing membranes and lipid droplets.

RESULTS

Figure 1 shows the proportion of cells in S in ascites tumours of various ages. The application of an analysis of variance [18] to these results indicated no significant difference with time from 3 to 21 days inclusive, (P = > 0·05) probably due to the wide scatter of ^{3}H-TdR labelling indices in the later stages of tumour growth. If the period 3 to 10 days inclusive was considered, the decline in the proportion of cells in S was just significant (P = < 0·05).

Thymidine kinase (TdR-kinase) activity in tumour cells in pairs of animals inoculated with cells from the same sequence of donors and

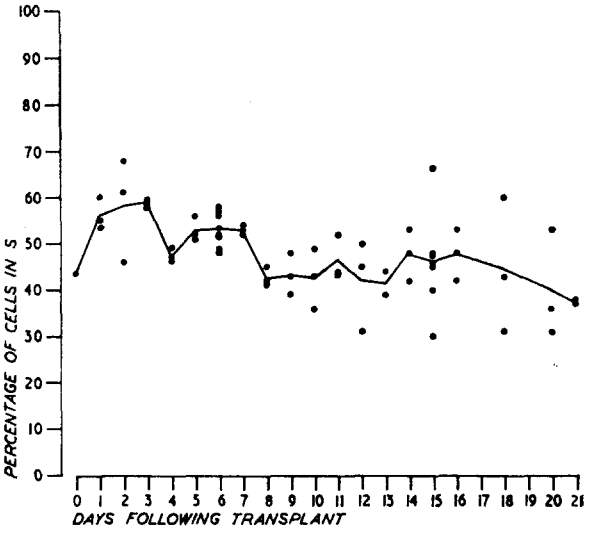

Fig. 1. *Percentage of cells in S. vs tumour age. Mean values joined by a continuous line. Heavy dots represent actual points.*

sacrificed at 6 and 15 days is shown in Table 1. The estimate of TdR-kinase activity was based on duplicate analyses of the same cell suspension.

The proportion of cells synthesizing DNA was significantly greater at 6 days than at 15 days (Student's t test, P = < 0·05).

Table 1. *Thymidine kinase activity* and labelling index*

6-day levels ± S.E.M.	Labelling index	15-day levels ± S.E.M.	Labelling index
1·309± 0·055	54·2	0·266± 0·015	40·0
2·250± 0·015	56·9	0·885± 0·075	30·0
0·392± 0·010	64·2	0·407± 0·016	66·6
0·435± 0·017	58·0	0·178± 0·012	45·0
0·705± 0·020	49·0	0·351± 0·013	47·7
0·272± 0·010	55·3	0·167± 0·007	46·0
0·251± 0·014	58·5	0·055± 0·009	48·2

*% TMP/min/10^6 tumour cells

Analysing these results, it can be shown that the TdR-kinase activity per tumour cell at 6 days was significantly greater than at 15 days (P = < 0·05, Wilcoxon test) [18]. The coefficient of correlation between the ^{3}H-TdR labelling index and TdR-kinase activity was not significant (P = > 0·2) [18].

Samples of peritoneal cells from control animals were analysed and found not to contain any detectable TdR-kinase activity or to donate any detectable protein to the fractions which normally included TdR-kinase.

Combining the supernatants containing the TdR-kinase extracted from 6- and 15-day

tumours and estimating the kinase activity, was found to give results predicted from the estimate of this enzyme in the two supernatants individually (Table 2). This suggests that it is unlikely that the reduction of TdR-kinase activity in the extracts from older tumours is due to the presence of excess, unattached or loosely-bound inhibitor associated with this enzyme.

To test the effect of increasing the level of availability of an electron acceptor, pyruvate [19] in 0·9% NaCl was injected into the peritoneal cavity to give a final concentration of 0·0012M. The ^{3}H-TdR labelling index, estimated 1, 2, and 6 hr following the injection, did not change. On the other hand, G6PDH activity rose sharply during the first 1 to 2 hr after injection of pyruvate and, in the majority of animals, was still high 6 hr later (Table 3). As a control, sodium acetate was injected to give the same molarity as pyruvate and this

*Table 2. Thymidine kinase activities**

Tumour age (days)	Individual activity (± SEM)	Combined		
		Predicted	Obtained	(± SEM)
6	0·223 ± 0·013	0·121	0·122 ±	0·008
15	0·019 ± 0·004			
6	0·192 ± 0·0069	0·132	0·131 ±	0·006
15	0·071 ± 0·013			
6	0·128 ± 0·011	0·146	0·130 ±	0·008
15	0·164 ± 0·010			

*% TMP/min/10^6 tumour cells.

Histochemical detection of dehydrogenase activity

SDH and LDH activity in cells from old (13 to 18 days) and young (4 to 8 days) tumours were essentially the same. At both these stages tumour cells showed some degree of individual variation in staining intensity which was not, apparently, correlated with cell size. This does not, however, rule out the possibility of correlation of staining intensity for a particular cell with regard to its position in the cell cycle.

A change was seen in G6PDH activity as the tumour aged. The staining intensity of this enzyme was strong in the 6-day tumours and barely detectable in cells from old tumours (17 to 20 days).

Eight tumours which had been growing for 14 to 22 days were aspirated and G6PDH activity estimated daily thereafter by histochemical methods together with the ^{3}H-TdR labelling index. The dehydrogenase activity increased considerably during the first 3 to 4 days following aspiration and was maximal shortly after the labelling index had reached its peak. Subsequently, as the total number of cells and ascitic volume increased, the labelling index and the G6PDH activity declined to a much lower level.

Table 3. Effect of pyruvate or acetate on glucose-6-phosphate dehydrogenase staining intensity

Tumour age (days)	Treatment	Hours after treatment (0	1	2	6)
		Staining intensity			
Old tumour (17–20)	Pyruvate*	0	2	1	3
		1	3	2	3
		1	2	3	2
		0	0	2	3
		1	2	0	1
Old tumour (17–21)	Acetate*	0	0	0	0
		1	1	1	1
		1	1	1	1
		1	1	1	1
		1	1	1	1
Old tumour (17–21)	None	0	0	0	0
		0	0	0	0
		1	1	1	1
		1	1	1	1
		1	1	1	1

*Given as i.p. injection in 0·5 ml 0·9% NaCl.

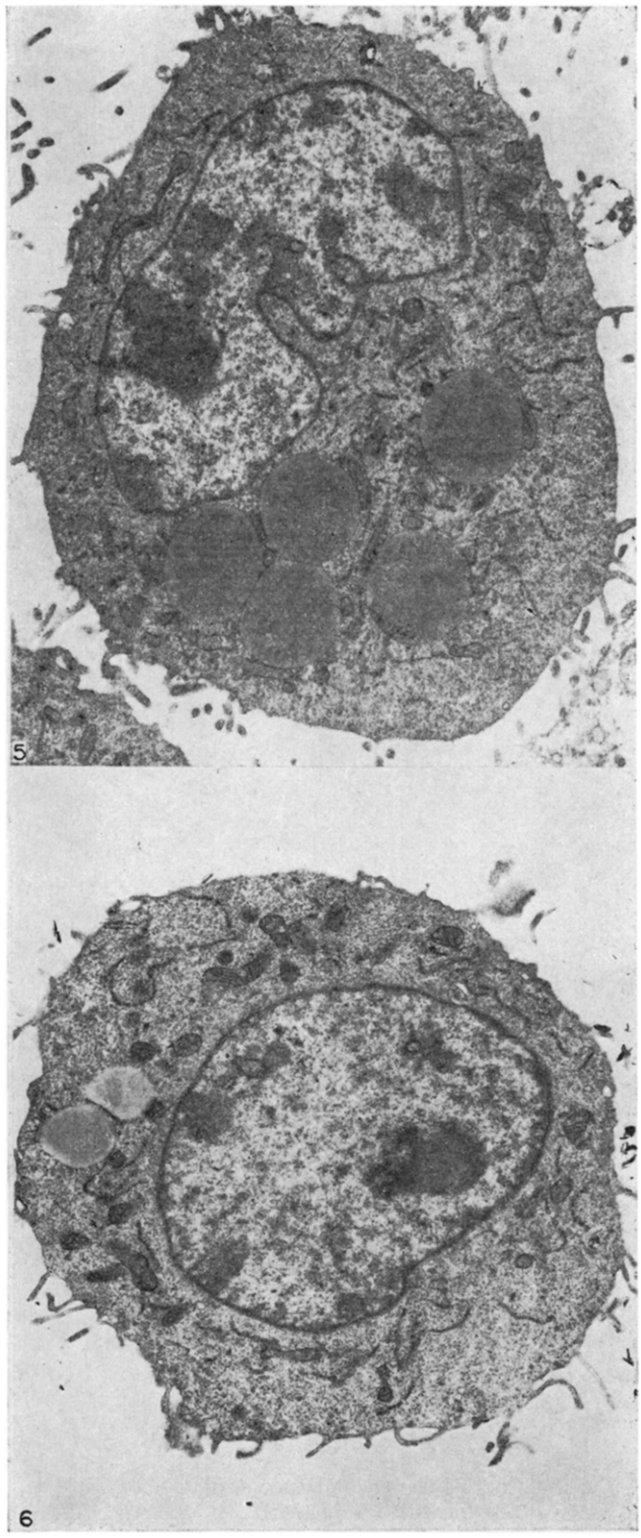

Fig. 5. *Electron micrograph showing tumour cell* 15 *days after transplantation.* (× 13,800)

Fig. 6. *Electron micrograph showing tumour cell* 5 *days after transplantation* (× 13,800)

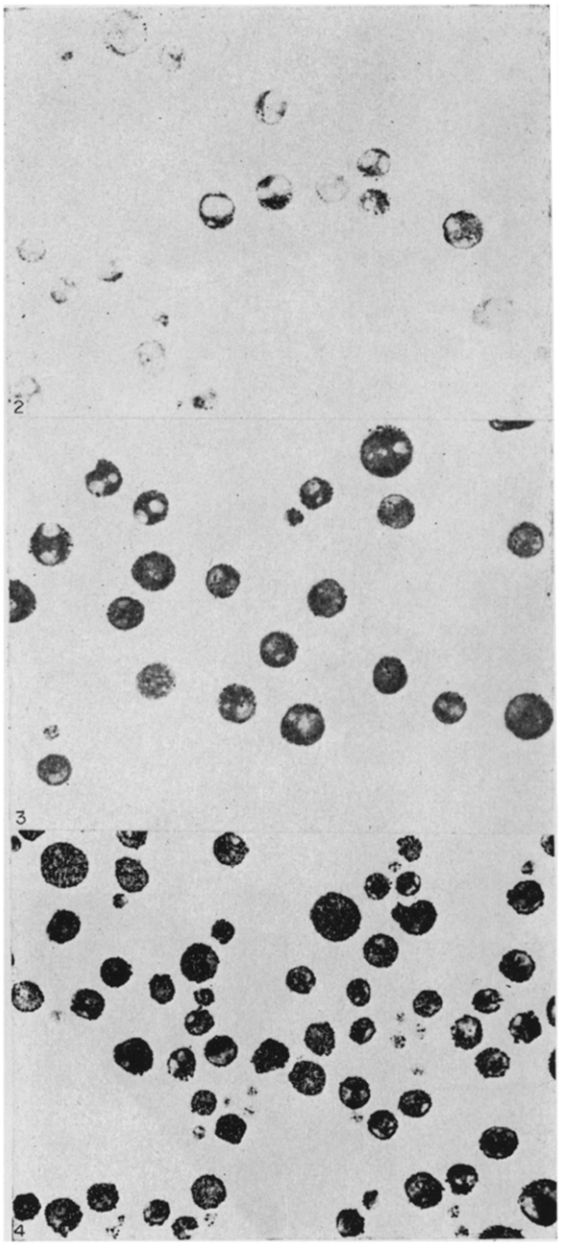

Fig. 2. *Ascites tumour cells from a* 15*-day tumour. Grade* 2 *G6PDH stain intensity.*

Fig. 3. *Ascites tumour cells from an* 8*-day tumour. Grade* 3 *G6PDH stain intensity.*

Fig. 4. *Ascites tumour cells from a* 6*-day tumour. Grade* 4 *G6PDH stain intensity.*

(to face p. 464)

procedure did not affect the labelling index or G6PDH activity. The incorporation of pyruvate or acetate into the incubation mixture for the detection of G6PDH activity in smeared cells had no noticeable effect on enzyme activity.

As histochemical recognition of G6PDH requires the presence of a diaphorase enzyme to catalyse the transfer of hydrogen ions from the reduced co-enzyme to the tetrazolium salt, the histochemical activity of this enzyme was compared and found to be identical in young and old tumours.

Biochemical assay of G6PDH activity

G6PDH activity in 6- and 17-day old tumours is shown in Table 4. When values are expressed in activity per minute for a given number of tumour cells, the ratio of G6PDH activity for a 17-day tumour to that for a 6-day tumour was generally greater than 1. However, this ratio decreased when samples were made more comparable by correction for the difference in mean cell volumes at these two ages. Discrepancies between the G6PDH activities as estimated biochemically and histochemically are clear.

Table 4. Glucose-6-phosphate dehydrogenase activity

Pair No.	6-day levels (biochemical)*	Labelling index	17-day levels (biochemical)*	Labelling index	Ratio of biochemical levels 17/6	Ratio corrected for volume†	Ratio of histochemical levels 17/6
(1) Difference method							
1	8·72	49·1	8·16	24·9	0·936	0·747	0/4
2	4·28	57·7	8·42	25·5	1·967	1·57	2/4
3	10·88	56·2	15·37	17·2	1·413	1·128	1/4
4	8·34	51·6	11·04	14·7	1·321	1·06	0/4
5	8·62	48·1	11·22	26·9	1·302	1·04	0/4
(2) Excess glucose-6-phosphate‡							
	(± SEM)		(± SEM)				
1	14·39 ± 0·072		14·50 ± 0·036		1·008	0·804	
2	8·13 ± 0·002		11·37 ± 0·219		1·399	1·117	
3	11·39 ± 0·191		19·46 ± 0·230		1·709	1·364	
4	10·64 ± 0·099		12·27 ± 0·301		1·153	0·920	
5	10·24 ± 0·152		17·97 ± 0·008		1·755	1·401	

*Δ OD $_{340}$/min/10^9 tumour cells.
†Mean cell vol (μ^3) ± SEM for 6- and 17-day tumours; 2083·6 ± 52·95 and 2610·3 ± 67·60 respectively.
‡Pair numbers, labelling indices and histochemical gradings correspond for both biochemical assay methods.

Ultrastructural and further histochemical studies

Ultrastructural examination of cells from older tumours showed these cells to contain a greater number of distinct lipid droplets than those from younger tumours (Figs. 5 and 6); this was confirmed histochemically. Mitochondria in cells from 15-day tumours showed abnormalities of the arrangements of the cristae, in particular the formation of cristae which were oriented parallel to the long axis of the mitochondrion, and also the presence of cystic spaces. The mitochondrial changes are, possibly, a reflection of increasing hypoxia coupled with general cell deterioration and ageing. The overall cytoplasmic density was lower for cells from older than for those from younger tumours, and there was a decrease in the quantity of rough endoplasmic reticulum in the former cells, which would be consistent with a decreased growth rate. These changes are similar to those reported previously [20, 21].

About 1% of cells were non-viable in tumours of 2 to 13 days growth; by 17 days this had increased to approximately 9%.

Staining with PAS preceded by diastase treatment failed to reveal the presence of glycogen in either young or old tumours. PAS-positive-diastase-resistant cytoplasmic granules, possibly lipid in origin, were present to the greatest extent in cells from tumours of more than 13 to 15 days growth.

Acid phosphatase activity was lowest in cells from very young tumours (2 days), reached maximum activity at about 6 days, and decreased to low activity in cells from old tumours (17 days). These changes may simply reflect different degrees of enzyme synthesis and breakdown. Inorganic phosphate is known to inhibit acid phosphatases [22] and the pH

of the ascitic fluid may also be important in this respect. Both of these factors may be inhibitory of high acid phosphatase activity and synthesis in very young tumours, because of the relatively high metabolic activity of the tumour, and similarly adverse in very old tumours because of the products of cell lysis.

DISCUSSION

This study has demonstrated a significant decrease in TdR-kinase activity per cell as the tumour grows. This finding is contrary to that obtained by Harris *et al.* [6], but they did not express the statistical significance of their observations.

TdR-kinase activity is raised in many animal tissues such as regenerating liver [23], foetal rat liver [24] and tumours [25] and gives an indication of proliferative activity. Hence the decline of the TdR-kinase activity is in keeping with a decrease in overall proliferative activity with increasing age of this Ehrlich tumour.

In the older tumours, the TdR-kinase containing fraction is extracted from actively proliferating cells as well as those that have not synthesised DNA for at least 48 hr (out of cycle) [5]. It is possible that the kinase activity per cell may be constant throughout tumour growth in actively proliferating cells but falls in those out of cycle and this would explain the overall decrease which was observed. The situation is somewhat analogous to that of liver in which lower kinase activity is found in adult as compared with regenerating liver [23].

There was no significant correlation between TdR-kinase activities and ^{3}H-TdR labelling indices. However, as the labelling index depends upon more than TdR-kinase activity alone, a simple correlation need not exist.

During the labelling process *in vitro*, a considerable proportion of the out-of-cycle population in tumours of 13 days and over could be lysed thus raising the apparent labelling index. Furthermore, it is possible that some of these out-of-cycle cells might commence DNA synthesis when transferred into the well-oxygenated culture medium *in vitro*. This may explain the large variation in the percentage of cells synthesising DNA that was observed particularly in the later phases of tumour growth (Fig. 1). Additionally, it is possible that in very large ascitic tumours, because of the necrotic deposits, a non-representative sample is withdrawn for the *in vitro* labelling index determination. The restriction of circulation of the ascites, caused by these latter deposits, renders *in vivo* labelling index determination similarly fraught with difficulties.

The decrease of overall proliferative activity with tumour age, as indicated by TdR-kinase activity and development of an out-of-cycle population, may be the consequence of the extremely low oxygen tensions found in large ascitic tumours [26], together with diminished frequency of random contact of cells with the peritoneum where oxygen is transferred from capillaries into the ascitic fluid [4]. Harris *et al.* [6] have implicated both of these factors to explain accumulation of cells in the post-mitotic, pre-DNA-synthetic period which corresponds to our out-of-cycle cells [5].

Histochemically, decreased G6PDH activity may support the general hypothesis that oxygen insufficiency is an important causative factor in decreased metabolism of older tumours since Biczowa *et al.* [27] have shown that Ehrlich ascites tumour cells are unable to grow under anaerobic conditions.

G6PDH occupies a key position at a branch point in metabolism and may be one of the control points in the pentose phosphate pathway [10] which in ascites cells appears to be mainly involved in the production of reduced coenzyme [19]. One factor limiting the oxidative reactions of the pentose phosphate path is the rate of reoxidation of NADPH [10]. Substrate limitation here, does not seem to play such an important role as coenzyme limitation. Both the dehydrogenases of this path are apparently subject to inhibition by NADPH [10].

This suggests that G6PDH would be particularly susceptible to inhibition by any build-up in reduced coenzyme. The production of oxidised coenzyme depends finally upon oxygen availability which falls progressively in ascitic tumours [26]. Oxygen supply to cells in an older tumour is further diminished by the accumulation of necrotic cellular debris. The decreased histochemical activity of G6PDH in older tumours could, therefore, be seen as reflecting an increase in the ratio of reduced to oxidised NADP resulting from increasing hypoxia.

Wenner *et al.* [19] showed that pyruvate stimulated the activity of the pentose phosphate pathway in ascites cells *in vitro* and concluded that pyruvate served as an electron acceptor for the NADPH generated by the initial enzymes of this path. They considered the conversion of pyruvate to lactate by an NADP-dependent lactate dehydrogenase was most probable.

The stimulatory effect of pyruvate on G6PDH activity supports the idea that a relative increase of NADPH in older tumours might be the reason for their low histochemical G6PDH activity.

The possible role of LDH in the mechanism proposed by Wenner and his colleagues for the stimulatory effect of pyruvate upon the pentose phosphate path, is supported in some measure by the work of Gumaa and McLean [28] who used oxamate to inhibit LDH in ascites tumour cells *in vitro*. There was a general decrease in pentose phosphate pathway intermediates, which they interpreted as being in keeping with an inhibitor of NADPH re-oxidation. However, the effect was not unequivocal as oxamate has the additional effect of lowering the hexokinase activity in ascites cells [29].

The rapidity of the stimulatory effect of pyruvate in the present studies, on G6PDH activity, may reflect activation of existing enzyme, together with the release of preformed enzyme from polysomes. Kaplan [30] has suggested that coenzyme levels may be important in this respect; oxidised coenzyme may be specifically required.

Although respiration is necessary for growth of this tumour, the cells derive the majority of their energy from glycolysis [31]. It might be expected, therefore, that fluctuations in SDH activity (an enzyme representative of the TCA cycle) would be difficult to detect histochemically. However, it would seem that neither SDH nor LDH are subject to inhibition by build-up in reduced coenzyme concentration.

Probably as a result of increased availability of oxygen and metabolic intermediates such as pyruvate, reduction of tumour mass by aspiration leads to an increase in the ^{3}H-TdR labelling index and histochemical activity of G6PDH.

Optimal conditions for lipid synthesis are provided by high concentrations of acetyl CoA (or its precursors plus generating systems) coupled with relatively low concentrations of oxalo-acetate (and its precursors) of the TCA cycle [32]. These conditions would tend to prevail towards the end of tumour growth when cells are existing under rising hypoxia and are increasingly reliant upon glycolysis for total energy production. Raised levels of reduced coenzyme would also favour lipid synthesis, however limited; certainly, raised levels of oxidised nicotinamide nucleotides have been suggested to inhibit lipid synthesis [33].

In contradiction to the histochemical findings, the results of biochemical estimation by two different methods, showed no significant difference in G6PDH activity in young and old tumours. However, the aspiration and pyruvate experiments strongly support the histochemical findings to be a true reflection of enzyme activity in the intact cell *in vivo*.

There are two explanations for the above discrepancy: firstly, the biochemical assay was made under more artificial conditions and, possibly indicates that the lower histochemical G6PDH activity in the older tumour reflects the presence of an inhibitor, possibly NADPH as suggested previously, which is removed or oxidised aerobically or by enzymes brought into contact with the NADPH by the altered spatial arrangement involved in the biochemical sequence. Secondly, it is possible that during the extraction process an appreciable amount of formed, but ribosome-attached enzyme, is released and activated.

If G6PDH activity had been assayed only by the described biochemical method, the significance of the constant specific activity with increasing tumour age would have remained hidden.

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Letter to the Editor

Inhibition of NADH-Linked Respiration with the Anti-Cancer Agent 4'-Demethyl-Epipodophyllotoxin Thenylidene Glucoside (VM-26).

MARIO GOSALVEZ, J. PEREZ-GARCIA AND M. LOPEZ

Bioquimica Experimental, Clinica Puerta de Hierro, Madrid, Spain

STÄHELIN has shown that the compound VM–26 is a powerful anti-cancer agent which inhibits the beginning of mitosis, and whose action depends on the time that the drug and cells are in contact [1].

We have studied the effect of VM–26 on respiration of isolated mitochondria, submitochondrial particles and suspensions of Ehrlich ascites cells, in order to determine whether VM–26 could exercise its antimitotic effect through the energy metabolism of the cell, as do some respiratory inhibitors [2, 3].

Rat liver mitochondria were isolated by the method of Scheneider [4]. Submitochondrial particles were prepared by freezing and thawing rat liver mitochondria. The respiration of the mitochondria and submitochondrial particles was determined polarographically with a Clark type oxygen electrode in a medium composed of 0·25 M sucrose, 0·02 M KCL, 7 mM MgCl, 5 mM Pi and 5 mM Tris-HCl (pH 7·2; 22°C). Stock suspensions of Ehrlich ascites tumor cells were prepared as described by Chance and Hess [5] and their respiration was polarographically determined in an isotonic saline phosphate medium [5]. VM–26 was added in aliquots not greater than 5 μl of a solution in dimethylformamide. The controls were run with the solvent alone.

Figure 1(a) shows the effect of VM–26 on rat liver mitochondria respiring with glutamate and malate in presence of ADP. When VM–26 is added to a 100 μmole concentration, respiration is rapidly and almost completely inhibited. This inhibition of respiration is abolished when succinate is added. Figure 1(b) lemonstrates the importance of the time factor in VM–26 inhibition. In this figure we see various superimposed respiratory records in which VM–26 has been added 2 min before ADP. It is observed that complete inhibition of respiration is achieved with various concentrations of VM–26 but when the concentration is lower, complete inhibition is only achieved after several minutes (15 min for 12·5 μM VM–26). In no case was the inhibition released by the uncoupler dinitrophenol. Figure 2(a) shows the inhibition induced by VM–26 on the respiration of rat liver submitochondrial particles respiring with NADH as substrate. The inhibition is abolished with succinate, as occurred in the case of isolated mitochondria. Figure 2(b) shows the inhibition induced by VM–26 on the respiration of Ehrlich ascites tumor cells respiring with endogenous substrate. The inhibition is complete 2 min after VM–26 is added to a 50 μM concentration, and is also released by succinate.

The respiratory inhibition produced in submitochondrial particles and Ehrlich ascites cells by VM–26 depends on time in a way similar to that observed in mitochondria. On the other hand, inhibition in the three systems for a given concentration of VM–26 is proportional to the amount of respiring material used (not shown). For complete inhibition of

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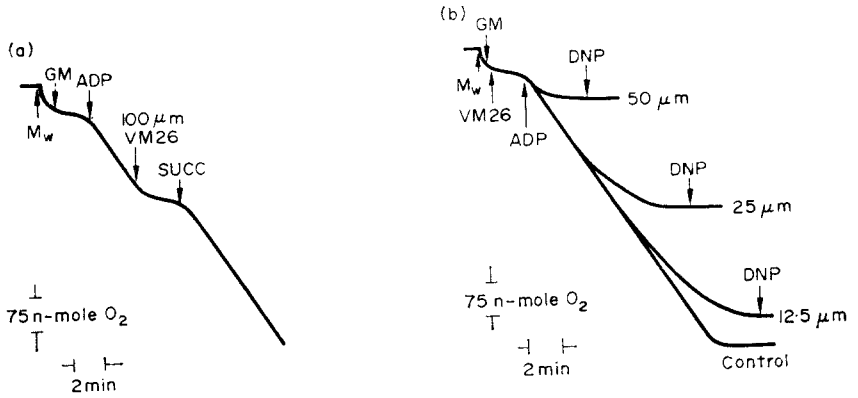

Fig. 1. *Time proceeds from left to right. Oxygen is consumed from top to bottom. The bottom horizontal part of the control record in* (b) *represents zero oxygen.*
(a) *Additions:* M_w, *Rat liver mitochondria,* 1·2 *mg mitochondrial protein/ml;* GM, 6 *mM Glutamate and malate;* ADP, 1 *mM ADP;* Succ, 8 *mM succinate.*
(b) *Various superimposed respiratory records are shown. Additions:* M_w, *Rat liver mitochondria,* 0·9 *mg mitochondrial protein/ml;* GM, 6 *mM glutamate; and malate;* VM–26, *VM–26 at the concentration indicated at the right of each record, except in the control where* 5 μl *of dimethylformamide were added;* ADP, 1 *mM ADP;* DNP, 70 μmol *dinitrophenol.*

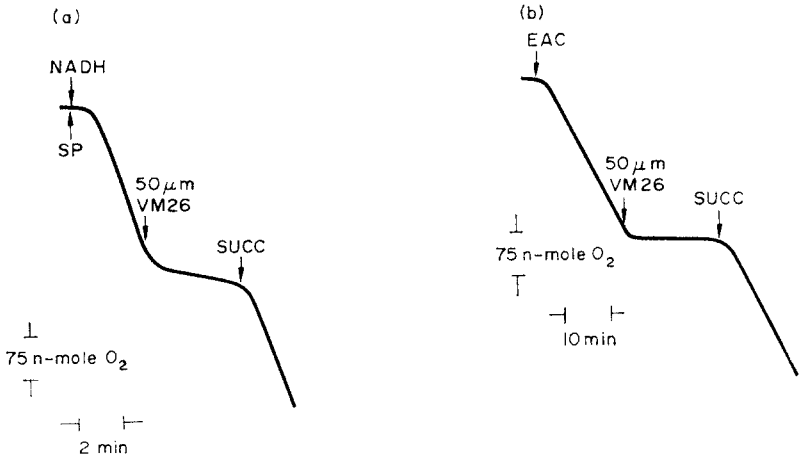

Fig. 2. *The same standard indications as in Fig.* 1.
(a) *Additions:* SP, *Rat liver submitochondrial particles,* 0·9 *mg mitochondrial protein/ml;* NADH, 1 *mM NADH;* Succ, 8 *mM succinate.*
(b) *Additions:* EAC, *Ehrlich ascites cells,* 15 *million cells/ml.* Succ, 8 *mM succinate.*

respiration in Ehrlich ascites cells in less than 20 min, 1 to 6 nmoles of VM–26 per million cells are required. (These figures represent the extreme values found in ten different preparations).

VM–26 seems to inhibit electron transport in the respiratory chain at the NADH dehydrogenase level, since respiration with succinate is not affected in any of the systems studied; nor is respiratory inhibition abolished by dinitrophenol. VM–26 inhibition is progressive in time, and more rapid with high concentrations of the drug or low concentrations of respiring material. This time dependency has also been indicated with regard to its antimitotic action [1].

The fact that complete inhibition of respiration by VM–26 is detected in a few minutes at concentrations only 5 to 20 times greater than those necessary to inhibit *in vitro* or *in vivo* cellular growth after incubations ranging from 3 to 24 hr (1 μM or 0·2 nmoles per million cells) [1, 6] would allow us to suggest that the antimitotic effect of VM–26 may come about through the inhibition of cellular respiration with substrates linked to NADH. This respiratory inhibition would prevent formation of the energy necessary in the premitotic period [2] and in the metaphase–anaphase transition [3]. The accumulation of metaphases observed with VM–26 in short incubation periods [1] would probably be due to a partial inhibition of respiration as had been observed for other respiratory inhibitors [3].

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Announcement

Second International Symposium on Cancer Detection and Prevention

Bologna (Italy), April 9–12th, 1973

The Second International Symposium on Cancer Detection and Prevention, promoted by the International Study Group on Detection and Prevention of Cancer (De P Ca), under the auspices of the International Union against Cancer (UICC) and of the International Agency for Research on Cancer (IARC) of the World Health Organisation, and organized by the Istituto di Oncologia "F. Addarii" and Bologna Cancer Center, will be held in Bologna from April 9 to 12, 1973.

The objectives of the Symposium are the discussion and the evaluation of the most recent knowledge and results in the field of cancer prevention and detection.

The program includes:

(1) Lectures on:
- Cancer prevention by environmental control
- Occupational carcinogenesis
- Host factors in oncogenesis
- Recent advances and perspectives of automation in cytology
- Aspects of experimental carcinogenesis related to cancer prevention
- Evaluation of cancer control measures (Report from the Sheffield UICC Meeting)
- Carcinogenic hazards from drugs
- The possible importance of nitrosamines as carcinogens in humans.

(2) Panels on:
- (A) Scientific bases and possibilities of detection and prevention of:
  - Cancer of the breast
  - Cancer of the lung
  - Gastric cancer
  - Cancer of the colon and rectum
  - Cancer of the urinary tract
  - Uterine cervix carcinoma.
- (B) Public Health aspects in Cancer Control.
- (C) Recent advances on tumours with particular racial and geographic distribution (oral cancer, Burkitt's lymphoma, nasopharyngeal cancer, esophageal cancer, liver cancer).

(3) Proferred paper sessions on the same topics as those of panels and on other fields of cancer detection and prevention.

Workshops for registered participants are planned the two days after the Symposium (April 13 and 14) on: epidemiological techniques, cell pathology, thermography, testing of environmental carcinogens, cytogenetics, needle aspiration cytology, gastric cytology.

Official languages: English and French.

Secretariat:
2nd International Symposium on Cancer Detection and Prevention
Istituto di Oncologia "F. Addarii"
Viale Ercolani 4/2–40138 Bologna, Italy. Phone 051–390417
Cable DEPCA–Bologna.

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Multiple-Drug Therapy in Hodgkin's Disease

R. D. RUBENS,* E. WILTSHAW,† E. BOESEN‡ and D. A. G. GALTON,§

The Royal Marsden Hospital and Chester Beatty Research Institute, Institute of Cancer Research, Fulham Road, London, S.W.3.

Abstract—*Thirty-seven patients with advanced Hodgkin's disease were treated with mustine, vincristine, procarbazine and prednisone administered in combination. The therapeutic effects were assessable in* 34 *patients.* 19 *obtained complete remissions ranging from* 6 *to* 36 *months (median* 23 *months), these being maintained without further treatment for periods of* 2–31 *months (median* 14 *months).* 14 *of these patients were still in remission at the time of analysis. Partial remissions were induced in a further* 14 *patients and only one derived no benefit. Severe toxicity was rare. In no case was it necessary to discontinue multiple-drug treatment because of bone-marrow suppression. Neurotoxic effects while common, were rarely severe:* 18 *patients had mild parasthesiae and* 7 *had objective muscle weakness, but this necessitated the discontinuation of treatment in only one case. It is essential that anti-emetic drugs are administered in dosage and frequency sufficient to be effective. The quality and duration of the remissions induced by multiple-drug therapy are superior to those obtained with single drugs or two-drug combinations.*

INTRODUCTION

EXPERIENCE in the treatment of lymphoblastic leukaemia both in animals [1], and man [2] has shown clearly the superiority of combinations of cytotoxic drugs over the same agents used singly. Lacher and Durant [3] used vinblastine and chlorambucil in combination in the hope of inducing remissions in Hodgkin's disease without incurring severe toxicity. Their remission rate of 63% was high compared with that usually reported for treatment with single agents, particularly as all but one of their patients had received previous treatment; Spurr *et al.* [4] had reported complete remissions in only 3% of previously treated patients compared with 26% in untreated ones.

Working on the basis that increased anti-tumour effects are obtained with several drugs administered in combination, de Vita *et al.* [5] used 4 agents which had different side effects. In this way they were able to give repeated courses with acceptable toxicity and they reported complete remissions in 12 of 14 patients (85%) with Hodgkin's disease [6]. Moreover the duration of remissions without maintenance therapy were in excess of 10 months in Stages 2b and 3.

We now report the results of treatment with a regime similar to that of Lowenbraun *et al.* [7] in patients with advanced and usually recurrent Hodgkin's disease. The study began in June 1968, and all patients with a diagnosis of Hodgkin's disease requiring chemotherapy were entered.

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*Medical Registrar, Royal Marsden Hospital.

†Honorary Consultant Physician, Royal Marsden Hospital. Senior Lecturer, Institute of Cancer Research.

‡Senior Lecturer, Institute of Cancer Research.

§Consultant Physician, Royal Marsden Hospital, Reader in Chemotherapy, University of London, Institute of Cancer Research.

Table 1.

Case No:	Sex	Histology	Previous treatment	Time: diagnosis to multiple drug therapy (months)	Symptoms	Extent of Disease			
						Node areas	Spleen	Lung	Other
1	2	3	4	5	6	7	8	9	10
1	F	NS	XC	26	3	—	—	—	—
2	M	NS	C	5	3	5	+	—	—
3	M	NS	XC	48	2	2	—	—	+
4	M	NS	XC	15	1	—	—	—	—
5	M	NS	XC	6	1	1	—	—	—
6	M	NS	XC	96	1	1	—	—	—
7	M	NS	XC	7	3	2	+	—	—
8	M	MC	X	25	2	2	—	—	—
9	F	NS	XC	117	3	1	—	+	—
10	M	LD	XC	48	3	—	+	—	±
11	F	LD	—	1	3	2	+	—	+
12	F	LH	XC	60	2	1	—	—	—
13	M	NS	XC	90	3	2	—	—	—
14	M	NS	XC	71	1	1	—	—	+
15	M	NS	XC	121	2	2	—	—	—
16	F	NS	XC	60	3	2	—	+	—
17	M	MC	X	4	3	3	+	—	—
18	M	MC	XC	12	4	3	+	—	+
19	M	MC	XC	48	3	2	+	—	—
20	F	LD	XC	48	1	—	—	+	—
21	M	NS	—	0	1	3	—	—	±
22	F	MC	X	35	2	4	+	—	—
23	F	NS	XC	37	1	1	—	—	—
24	M	LD	—	0	2	2	+	—	+
25	M	LD	X	1	1	—	—	—	—
26	M	NS	X	12	3	2	—	—	—
27	M	LD	XC	48	3	4	—	+	—
28	M	MC	C	10	2	4	—	—	—
29	M	NS	X	3	2	2	—	—	—
30	M	NS	XC	24	1	—	—	—	+
31	F	NS	X	65	2	1	—	—	—
32	M	LH	C	1	2	—	—	+	—
33	F	NS	X	52	1	1	—	+	—
34	F	—	XC	21	2	4	+	—	+
35	M	MC	X	20	3	2	—	—	+
36	M	NS	C	19	3	2	—	—	—
37	F	NS	XC	29	2	—	—	+	—

Column 3 NS = Nodular Sclerosis
MC = Mixed Cellularity
LD = Lymphocyte Depleted
LH = Lymphocytic-histiocytic

Column 4 X = Radiotherapy
C = Chemotherapy

Column 6 The numbers indicate the severity of symptoms. See text.

Column 7 The numbers indicate the number of node areas involved, eg 4 = 4 node areas. See text.

Number of courses	Duration of 6 courses in days	Remission achieved	Overall remission (months)	Unmaintained remission (months)	Follow-up in months to September 1971	Clinical state September, 1971
11	12	13	14	15	16	17
3	—	P	NA	NA	25	Rem (P)
6	183	*C*	18	13	20	Rem
6	184	*C*	23	8	25	Rem‡
6	302	NA	NA	NA	35	Rem
2	—	*C*	36	22	38	Rem*
6	217	*C*	33	26	35	Rem
5	—	P	NA	NA	17	Rem
3	—	*C*	19	13	20	Rem*
6	295	*C*	12·5	4	21	Rem (P)
6	178	P	1·5	0	8	Dead
4	—	P	3·5	0	8	Dead
6	293	*C*	25	17	26	Rem
3	—	P	1·5	0	4	Dead†
6	270	P	9	3·5	31	Rel
6	132	*C*	6	3	38	Rem
5	—	P	1	0	11	Dead
6	178	*C*	29	24	26	Rem
2	—	P	1	0	2	Dead
4	—	*C*	20	18	40	Rem (P)
4	—	P	25	23	26	Rem
3	—	P	NA	NA	38	Rem
6	217	*C*	23	16	25	Rem
6	224	*C*	21	14	22	Rem
6	258	*C*	11	3	17	Rem (P)
6	411	0	0	0	16	Dead
6	205	P	4	1	26	Rem (P)
6	200	P	6	< 1	12	Dead
2	—	P	4	0	22	Dead
6	176	*C*	36	31	37	Rem
6	172	*C*	10	2·5	13	Dead
6	206	*C*	15	10	17	Rem
2	—	P	1	0	3	Dead
4	—	*C*	28	22	30	Rem*
6	168	*C*	25	22	27	Rem
3	—	*C*	26	11	27	Rem*
1	—	NA	NA	NA	25	Rem (P)
½	—	NA	NA	NA	28	Rem (P)

Column 13 P = Partial remission
C = Complete remission
O = No remission

Column 14 NA = Not assessable

Column 17 Rem = Still in remission
Rel = Has relapsed
Rem (P) = Partial remission only.

* Courses of chlorambucil in addition to courses of multiple-drug therapy.

† Node removed before multiple-drug chemotherapy showed a lymphocyte depleted pattern.

‡ Has received 2 further courses of multiple drug therapy but has never relapsed.

Patients

Thirty-seven patients (25 males and 12 females) started treatment with the regime between June 1968 and October 1970. Histological material taken at the time of the initial diagnosis was reviewed by Professor N. F. C. Gowing or Dr. I. M. Hamlin, in all but one case, and classified by the method of Lukes and Butler [8]. However, in case 13, a second biopsy specimen obtained before multiple-drug therapy was started showed that the histological picture had changed from the nodular sclerotic to the lymphocyte-depleted type.

The intervals from the time of diagnosis to the commencement of multiple-drug therapy (Table 1) ranged from 0 to 126 months (median 25 months). Thirty-four (92 %) of the 37 patients had had previous treatment; either radiotherapy (9 cases), chemotherapy (4 cases) or both (21 cases.)

Staging (*Table* 1)

The extent of the disease in each patient before the commencement of multiple-drug therapy was assessed both clinically and radiologically (chest X-ray and lymphogram). Symptoms, especially fever, sweating and pruritus, were recorded. In Table 1, symptoms are graded 1 to 4 according to the extent of disability:

1. No symptoms.
2. Symptoms present but leading a normal life.
3. Symptoms sufficiently severe to restrict normal life.
4. Confined to bed because of the severity of symptoms.

The involvement of up to 6 groups of lymph nodes, (neck, axillae, mediastinum, para-aortic, pelvic and inguinal) is recorded in column 7 (Table 1), the maximum possible rating being 6. We have not classified our patients by the accepted method used for clinical staging at the time of presentation because it is not relevant when the distribution of the lesions has been greatly altered by previous treatment. For example, patients 1, 9, and 25 had no measurable tumour masses at the start of multiple-drug therapy, but their symptoms had recurred and the erythrocyte sedimentation rate (ESR) had risen. Furthermore, several patients who at presentation had Stage 2 disease received radiotherapy and the disease later recurred outside the irradiation field. Staging could have been based on the extent of the disease at that time alone, or in addition, on the areas previously involved. For example, patient 26 initially had disease confined to lymph nodes above the diaphragm (Stage 2). Twelve months after radiotherapy with the upper mantle technique, there was no recurrence in the irradiated areas, but the para-aortic and pelvic nodes were now enlarged. Should this patient be classified now as having Stage 2 or Stage 3 disease?

Treatment regime

The aim was to give each patient six courses of multiple-drug treatment. The standard doses were as follows:

1. Mustine hydrochloride (HN2), 6 mg./m^2 into the tubing of a fast running intravenous saline infusion on day 1 and day 8.
2. Vincristine, 1·4 mg./m^2 intravenously on days 1 and 8 (maximum dose 3 mg.).
3. Procarbazine, 100 mg./m^2/day by mouth on days 1–10 inclusive.
4. Prednisone, 40 mg./m^2/day by mouth on days 1–14 inclusive during courses 1 and 4 only.

Many patients experienced malaise and nausea, and some vomited repeatedly in the 24 hr following the injections. The symptoms were controlled in varying degree by the administration of a phenothiazine at regular intervals. Some patients preferred to be admitted for the night following each injection.

An interval of at least 14 days was left between courses to permit recovery of the bone marrow. If at the end of 14 days rest the leucocyte or platelet counts were low it was considered better to wait for the counts to recover and to give full doses of the drugs than to give the next course at lower dosage without waiting. Most courses were, however, given between 14 and 28 days of each other. On day 8 of any one course the dose schedule was modified if the total leucocyte count (W.B.C.) had not recovered to 4,000/μl., or the platelet count to 100,000/μl. according to the following scheme (Table 2).

By 31st December 1970, 20 patients had completed six courses of treatment, in all but one case (patient 25), within 10 months of starting. In 17 other cases, the treatment was discontinued before six courses had been administered. The longest follow-up since the completion of six courses of treatment was 31 months.

Table 2.

Leucocyte or platelet count/μl	Dose of drug % of standard			
	Prednisone	Vincristine	HN2	Procarbazine
W.B.C. 3,999–3,000	100	100	50	50
W.B.C. 2,999–2,000 and/or Platelets: 50–100,000	100	100	25	25
W.B.C. 1,999–1,000 and/or Platelets < 50,000	100	50	None	None
W.B.C. < 1,000	100	None	None	None

Assessment of response to treatment

Three categories of response were considered, namely, complete remission, partial remission, or failure. These are defined as follows:

Complete remission—disappearance of all symptoms and signs of disease. In addition, the ESR, serum transaminases and alkaline phosphatase levels must be normal.

Partial remission—considerable but incomplete regression of lesions with complete or almost complete relief of symptoms.

Failure—little or no improvement in symptoms or signs of disease or progression of disease.

Many patients responded after the first or second course of treatment. The period from the time of best response until the first sign of relapse is designated "overall remission". "Unmaintained remission" is the period from the end of the last course of chemotherapy to the time when relapse is first noted.

RESULTS

Therapeutic effects (*Table* 1)

The aim was to administer 6 courses of treatment. Two of the 37 patients (cases 36 and 37) received only one course; in one of them further treatment was suspended because of haematemesis from a simple gastric ulcer, in the second because of unacceptable vomiting. A third case (No. 4) cannot be evaluated because multiple-drug therapy was started after a course of radiotherapy had brought about complete regression of the only known foci (which were extranodal) of active disease (no recurrence 26 months later). Nineteen of the remaining 34 patients completed 6 courses of multiple-drug therapy.

Fifteen patients who received less than six courses of multiple-drug therapy. In 8 cases the treatment was abandoned because of relapse, but 7 other patients were in remission when the treatment was abandoned for various reasons.

Eight patients who relapsed during treatment. Seven patients obtained partial remissions after the first or second course but relapsed within a few weeks (cases 18, 32, 28) or between the 3rd and 5th courses (cases 7, 11, 13, 16). Six of the seven died within 11 months of starting the treatment, and the 7th (case 7) was moribund in December, 1970. In the 8th case (No. 1) a partial remission was obtained, but pulmonary infiltrations were found after the 3rd course, and multiple-drug therapy was abandoned in favour of local radiotherapy.

Seven patients in remission when treatment was abandoned. Two patients (cases 8 and 35) went abroad after receiving 3 courses when they were in complete remission. These, and two others who refused further multiple-drug treatment because of vomiting (cases 5 and 33) after the 2nd and 4th courses, subsequently received treatment with chlorambucil in 14-day courses at daily doses ranging from 15 to 20 mg until a combined total of 6 courses of treatment had been administered. All 4 patients remained in complete unmaintained remission on September 30th, 1971 after 11, 13, 22 and 22 months, and in overall remission after 19, 26, 28 and 36 months. A fifth patient (case 19) refused further multiple-drug therapy after the 4th course, because of vomiting. He was then in complete remission. One intravenous injection of cyclophosphamide (1g) was also poorly tolerated and further treatment was refused. He remained in remission for a further 18 months (overall remission 20 months) before relapsing. A sixth patient (case 20) was in partial remission after the 4th course but further treatment was not given because of recurrent chest infections. She remained without evidence of active disease, other than a raised erythrocyte sedimentation rate, when the study was ended 23 months later.

Finally, treatment was abandoned in one case (No. 21) after 3 courses because of severe neurotoxicity. The disease, then apparently confined to the mediastinum, was treated successfully by radiotherapy and there was no evidence of recurrence more than 3 years later.

Nineteen patients who received six courses of multiple-drug therapy. 14 patients remitted completely, 4 partially, and 1 patient failed to respond.

Ten of the 14 patients who remitted completely were still in remission on September 30th, 971, after periods up to 31 months after the last1 course; and they had been in overall remission from 15 to 36 months. Three patients relapsed within 3 months of the end of the 6th course (cases 15, 24 and 30). One (No. 30) died shortly after; another (No. 15) received a second series of six courses of multiple-drug therapy, but the ESR remained elevated at the end of the last course and received radiotherapy to the para-aortic region. The third (No. 24) has since gone into complete remission on weekly vinblastine.

The 4 patients (cases 10, 14, 26, 27) whose remissions were incomplete all relapsed within 3½ months of the end of the 6th course and two (cases 10 and 27) died shortly after.

Summary of all patients who obtained complete remissions (*Fig.* 1.)

Of the 19 cases, 14 patients completed 6 courses of multiple-drug therapy; in 5 cases the treatment was changed or abandoned after 2 to 4 courses had been administered. Fourteen of the 19 patients (including four of the five who did not complete 6 courses of treatment) were still in unmaintained remission on September 30th, 1971.

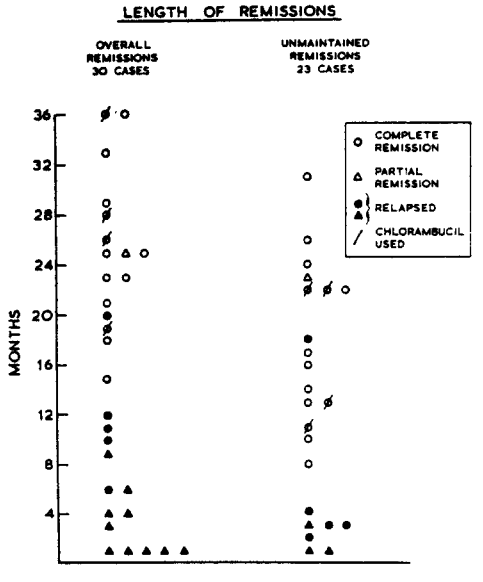

Fig. 1. *Duration of overall remission in* 30 *cases of Hodgkin's disease treated by multiple-drug therapy. Terms relating to remission are defined in the text. In* 23 *of the patients remissions continued without further treatment.*

Response in relation to histological type. The number of cases is too small to permit the recognition of the effect of histological type on the response rate except in the lymphocyte-depletion group. Only one of the six patients in this group remitted completely, compared with 17 of the 27 patients diagnosed as having the nodular sclerotic, mixed cellularity, or lymphocytic–histiocytic histological types.

Response in relation to previous treatment. Only 3 of the 34 patients had received no previous treatment, 3 had received chemotherapy, 9 had received radiotherapy, and 19 both chemotherapy and radiotherapy. The proportion of complete remissions was lower in the last mentioned group of patients than in the group treated previously by radiotherapy alone, and this reflects the higher proportion of patients with advanced disease. However, 6 of the 10 patients in the whole series who had the best response and who have remained in remission without further treatment for the longest periods had received previous treatment with both radiotherapy and chemotherapy, the other 4 having received radiotherapy only.

Toxicity

The side effects of each of the drugs used in this combination were not increased disproportionately when the agents were administered together.

Potentially the most dangerous effect was on the bone marrow. Falls in the haemoglobin of more than 2·0 g/100 ml occurred in 15 cases, and 4 patients required blood transfusions; after the courses were finished the haemoglobin rose to normal levels. Falls in the platelet counts to less than 80,000/μl were recorded in 22 cases. A leucocyte count of less than 1,000/μl was seen only once. In no case was it necessary to discontinue multiple-drug treatment because of suppression of the bone marrow.

Neurotoxic effects were recorded in almost all the patients but were annoying rather than disabling in most of them (48 % of patients had mild parasthesiae of the fingers, toes, or both). Objective evidence of muscle weakness was recorded in 7 patients but the treatment had to be stopped in only one case.

Multiple-drug therapy was postponed following the first course which appeared to provoke an attack of mania in a known manic-depressive patient (case 1), but two further courses without prednisone were subsequently administered without difficulty. One patient, known to have had a gastric ulcer, had a haematemesis after the first course (case 37). In both instances the prednisone might have contributed to the complications. In another case (Case 20), therapy was stopped after the fourth course because two attacks of pneumonia were thought to have been associated with treatment-induced leucopenia. This patient had irradiation-induced lung damage.

Four patients refused further multiple-drug treatment because of nausea and vomiting. More attention has since been paid to the details of anti-emetic treatment; two of these patients have accepted further courses of multiple-drug therapy without incident, and no other patient has refused further treatment because of side-effects in the last year. It is essential that anti-emetic drugs are administered in doses sufficient to be effective, even if these also induce drowsiness, and that the doses are repeated at regular intervals during the 24 hours following the injection. No patient developed jaundice or dystonic reactions to these drugs.

DISCUSSION

Recent reports on the treatment of advanced Hodgkin's disease [7, 9] have shown that repeated courses of treatment with several drugs administered simultaneously are far more effective than treatment with single agents [4, 10, 11]. Our results confirm these findings. Complete remissions were obtained in 19 of 34 patients (56%) all but three of whom had received previous treatment with radiotherapy (9 cases), chemotherapy (3 cases), or both (19 cases) for periods of 1 to 126 months (longer than 2 years in 19 cases). So far 8 patients have been in complete remission for more than 2 years.

It is possible that the superiority of the multiple-drug treatment is due to the large total dose of the cytotoxic drugs administered in a relatively short period, rather than to a synergistic effect of the drugs used in the combination. Two to four 14-day courses of chlorambucil at a daily dose of 0·4 mg./kg. were used to complete the planned series of 6 courses of multiple-drug therapy for four patients who for various reasons received only 2, 3, 3 and 4 courses. These patients responded as well as those who completed 6 courses. However, the preliminary results of a co-operative trial in which one schedule involves the administration of chlorambucil in 7-day courses [12] 2 to 3 weeks apart, in daily doses of 0·4 to 0·5 mg/kg suggests that the remission rate is low and the bone-marrow toxicity prohibitive. It is likely that therapeutic efficacy comparable to that obtained with multiple-drug therapy could not be obtained with any single agent now available without incurring insupportable toxic effects.

Our multiple-drug regime is essentially that of Lowenbraun *et al.* [7]. Nicholson *et al.* [9] introduced two main modifications; they replaced vincristine by vinblastine, and they included prednisolone in every course instead of omitting it from four of the six planned courses. Vinblastine is usually regarded as more effective than vincristine in Hodgkin's disease, but it is far more myelotoxic. Vincristine was incorporated instead of vinblastine in the protocol of Lowenbraun *et al.* to avoid the addition of a third myelotoxic drug to the two (mustine and procarbazine) already included. Nicholson *et al.* [9], however, found the neurotoxic effects of vincristine unacceptable. We replaced vincristine by vinblastine after the third course in only one case (No. 23) because of the severity of neurotoxic effects. We now prefer to limit individual doses of vincristine to 2 mg.

The role of prednisone in the combination is not clear, and our patients have not commented on subjective differences in those courses in which the steroid was omitted. One manic-depressive patient had an attack of acute mania during a course in which prednisone was administered, but remained well when she later received two courses without prednisone. Nicholson *et al.* [9] include prednisolone in every course, but they administer 40 mg to all patients believing that the dose of 40 mg/m^2 advocated by Lowenbraun *et al.* has been responsible for psychiatric disturbances. Two of our patients developed severe herpes zoster following courses incorporating prednisone, and it would be important to know whether the addition of a corticosteroid to every course will increase the incidence of viral or other infections.

The choice of 6 courses of multiple-drug therapy is arbitrary and an alternative approach would be to continue the courses for a fixed period of 12 or 18 months by analogy with the treatment of choriocarcinoma, in which the necessity for continuing treatment long after the disappearance of all clinical and radiological evidence of residual disease has been related to the persistence of minimal levels of

urinary chorionic gonadotrophin, indicating the presence in the body of about 1 million tumour cells [13]. The practical difficulty is to convince patients who now feel well of the need for continuing the intensive and sometimes unpleasant treatment that they were prepared to accept when they felt ill and from which they were deriving obvious benefit. Most of the patients are young and leading active lives. The quality and duration of the remissions induced by multiple-drug therapy are superior to those which were usually obtained with single-drug therapy. No doubt further improvement will result from refinements of the method.

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Results of a Clinical Trial with Intermittent Doses of Adriamycin in Lung Cancer

Y. KENIS, J. MICHEL, R. RIMOLDI, L. ISRAËL and P. LÉVY

*Cooperative Group for Clinical Trials in Lung Cancer (EORTC)**

Abstract—*Sixty patients with far-advanced lung cancer (epidermoid carcinoma: 38 cases; anaplastic small cell carcinoma: 15 cases; anaplastic large cell carcinoma: 2 cases; glandular carcinoma: 2 cases; histology not specified: 3 cases) were treated with an intermittent dose schedule of adriamycin. The drug was given i.v. at a dosage of 75 mg/m² every 3 weeks. Among 50 evaluable cases (at least 2 doses), there were 16 partial remissions, 3 partial failures and 31 total failures. The frequency of objective responses was similar among the different histological classes. The 90-day survival was 68% for all cases and 89% for the responders. The longest remission had a duration of 9 months (median: 3 months).*

Alopecia was practically constant. Bone marrow depression was frequent but almost always reversible. No clinical cardiac toxicity was observed but creatine phosphokinase was increased above normal values in 4 among 24 evaluable cases. The effect on delayed hypersensitivity was minimal. Stomatitis was exceedingly rare with this dose schedule.

Three cases died from progressive brain metastases while in continuing remission for the other lesions suggesting that adriamycin does not cross the blood brain barrier.

By comparing the results obtained by our group with daunorubicin and adriamycin, this latter drug seems more efficacious in lung cancer at a dosage which produces less severe toxicity.

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Participant members: R. Israël-Asselain, J. Chebat–Hôpital Foch (Suresnes); C. Molina, J. C. Cheminat–Hôpital Sabourin (Clermont-Ferrand); J. E. Couette–Centre François-Baclesse (Caen); R. Depierre, M. Jais–Hôpital Paul-Brousse (Villejuif); L. Israël–Hôpital Lariboisière (Paris); Y. Kenis, J. Michel–Institut Jules Bordet (Bruxelles); J. Le Melletier–Centre hospitalier (Versailles); H. Maisin, J. Longueville–Centre des Tumeurs (Louvain); A. Meyer, G. Nadjar–Hôpital Boucicaut (Paris); R. Rimoldi–Clinica Tisiologica (Milano); C. Sacerdoti–Ospedale Maggiore (Milano).

THE PURPOSE of this trial (a type I trial according to the terminology of the EORTC) was to evaluate the effect of adriamycin† administered intermittently in patients with lung cancer.

The histological diagnosis was epidermoid carcinoma in 38 cases; anaplastic (oat cell) carcinoma in 15 cases; anaplastic large cell carcinoma in 2 cases; glandular carcinoma in 2 cases; and not specified in 3 cases. All patients had far-advanced lung cancer and were not suitable for surgery or radiation therapy, according to the usual criteria of the Group [1, 2]. There were 58 males and 2 females in this group of patients. The median age at the

†Adriamycin was generously supplied by Farmitalia (Milan).

start of treatment was 58 years (range: 42 to 73 years). Previous treatments are listed in Table 1.

Table 1.

Previous therapy	
None	24 cases
Surgery	2 cases
Radiation therapy (RT)	15 cases
Chemotherapy (CT)	4 cases
Surgery + RT	5 cases
Radiation therapy + CT	8 cases
Surgery + CT	1 case
Surgery + RT + CT	1 case

The objective criteria for evaluation of response were as follows:

1. *A complete remission* corresponded to the complete disappearance of all signs and symptoms of the disease, for at least one month.

2. *A partial remission* was recorded when the decrease in measurable lesions was greater than 50% in the product of two diameters without progression of any other lesion and without appearance of any new lesions, during a period of at least one month; or a marked and unequivocal decrease (3/4 or more) of non-measurable lesions.

3. *A non-total failure* was characterized by a decrease of the lesions as in 2, but during a period of less than 1 month; or a definite decrease in some lesions while others were progressing.

4. *A total failure* was defined when there was progression of disease, absence of response, or any response smaller than 2 or 3.

Subjective improvement alone was not considered as a positive response. Patients who received less than 2 doses were considered as non-evaluable.

Adriamycin was given intravenously at a fixed dose of 75 mg/m^2 every 3 weeks. This dose was chosen according to the pharmacological data obtained by di Fronzo *et al.* with tritiated adriamycin [3]. The number of doses varied from 1 to 11, the majority of patients receiving 3 to 6 doses.

Before the treatment and before each dose, kidney and liver functions were tested by several biochemical determinations. EKG and a dosage of creatine phosphokinase (CPK) were performed at the same interval. Hemoglobin, white cell and platelet counts were carried out at least once a week during the course of treatment. Delayed skin sensitivity was tested before the treatment and periodically thereafter with 10 i.u. of PPD tuberculin intradermally. The lecture was made after 48 to 72 hr and read as positive when there was a papula of at least 5 mm in diameter.

RESULTS

1. *Antitumour effects*

Ten patients received only one injection and were considered as non-evaluable for the antitumour effect.

Objective results are listed in Table 2. No complete remission was observed. In a case of small-cell anaplastic carcinoma there was a complete disappearance of a subcutaneous metastatic nodule while the previously irradiated pulmonary lesion was unchanged.

A partial remission was observed in 15 other cases. Varied localizations of the malignant disease were responding to the treatment: the primary tumours (11 cases); subcutaneous metastatic nodules (3 cases); peripheral lymphadenopathies (2 cases); a pleural effusion and a superior vena cava syndrome (1 case). The changes in the primary tumour were considered as a partial remission when there was a marked decrease of a pulmonary and/or mediastinal mass or a very important (almost complete) decrease of atelectasia (3 cases). In one case, a large mediastinal mass rapidly progressing after

Table 2.

Histological class	Objective responses (No. of cases)					
	Non-evaluable	Partial remission	Non-total failure	Total failure	Total evaluable	Total
Epidermoid ca.	7	11	1	18	30	37
Anaplastic (small cell) ca.	1	3	2	9	14	15
Anaplastic (large cell) ca.	0	2	0	0	2	2
Glandular ca.	1	0	0	2	2	3
Not specified	1	0	0	2	2	3
Total	10	16	3	31	50	60

radiation therapy (6,350 rads) remained unchanged during 6 months after the beginning of adriamycin. According to our criteria this was recorded as a total failure but it is very likely that adriamycin had an important antitumour effect in this case. Three responders died from cerebral metastases without evidence of progression of the other lesions.

There was no obvious difference in the frequency of responses between the different histological classes. Eleven partial remissions were obtained among 30 evaluable patients with epidermoid carcinoma and 5 among 16 for the anaplastic carcinomas.

The median survival time from the beginning of chemotherapy was 200 days for the responders (partial remissions and non-total failures) and 162 days for the non-responders, the patients who have received only one dose being excluded. The number of responses was greater and the median survival was longer among the patients who had no prior therapy. The survival time was the same for the responders and the non-responders among the patients with prior therapy (Table 3). The 90-day survival was 68% for all cases (including patients who received only one dose), and 89% for the responders.

Table 3.

Median survival, in days, for the patients*

	Without prior therapy	With prior therapy	All cases
Responders	220(11)†	112(8)	200(19)
Non-responders	190(12)	112(19)	162(31)
Total	210(23)	112(27)	169(50)

*Non-evaluable cases have been excluded.
†Between brackets: number of cases.

The longest remission had a duration of 9 months (median : 3 months; mean : 4·3 months).

2. *Toxicity*

The most frequent side-effect was alopecia (Fig. 1). It was usually almost complete and was observed in 47 patients who received at least 2 injections (94%). It was reversible in the patients who were followed long enough (2 or 3 months) after the discontinuance of the drug.

Vomiting and/or nausea were present in 24 cases. This side-effect was not very troublesome with this intermittent dose schedule. Three patients had diarrhea. Stomatitis was observed in 2 cases, after the first and after the third dose, respectively. A skin rash appeared after the 4th dose in one case.

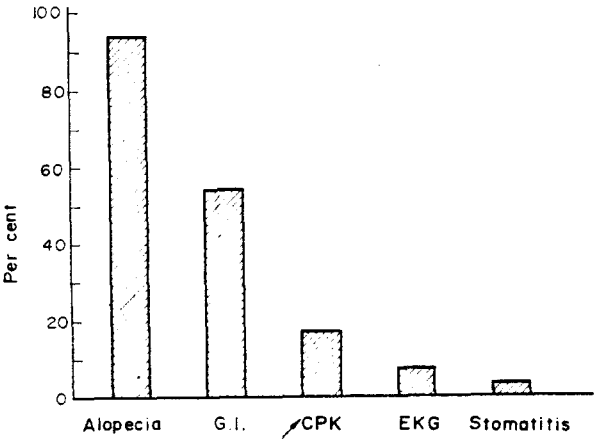

Fig. 1. *Frequency of toxic manifestations* (GI: *gastro-intestinal disturbances;* ↗CPK: *increase in creatine phosphokinase;* EKG: *EKG abnormalities*).

Clinically there were no signs of cardiac toxicity. However, CPK was increased above the normal values in 4 cases (among 24 evaluable cases) and was accompanied by a first grade atrioventricular block in one case. One patient had a transient partial left bundle branch block. No other EKG abnormalities were observed.

The tuberculin skin test was negative before the treatment and remained negative in 4 cases. It was positive and remained positive (with the same intensity) in 17 cases. It became negative during the treatment in 3 cases. A negative test became positive after 3 doses in one case.

Hematologic toxicity was usually not severe and was almost always reversible within 3 weeks (Fig. 2). Leukopenia (less than 3000 leukocytes/mm^3) was observed in 27 among 30 evaluable cases. Severe leukopenia (less than 1000) was present in 4 patients. Thrombocytopenia (less than 100,000 platelets/mm^3) was less frequent : 12 cases among 29. The platelets fell below 30,000/mm^3 in 3 cases.

The median nadir of the leukocytes was on day 11, and the nadir of the platelets on day 8 (Fig. 3 and 4). There was no cumulative toxicity (Fig. 2).

A reversible decrease in the reticulocytes and a concomitant increase of the serum iron level was observed in 14 cases where these parameters were studied (Fig. 5). However anemia, defined as a fall of more than 20% of the pre-treatment value of hemoglobin, was rare (9/39).

Toxicity may be accounted as the cause of death in one case; this patient died 6 days after the first dose of adriamycin, with a severe leukopenia (350 leukocytes) and infection. The blood count was normal before the treatment.

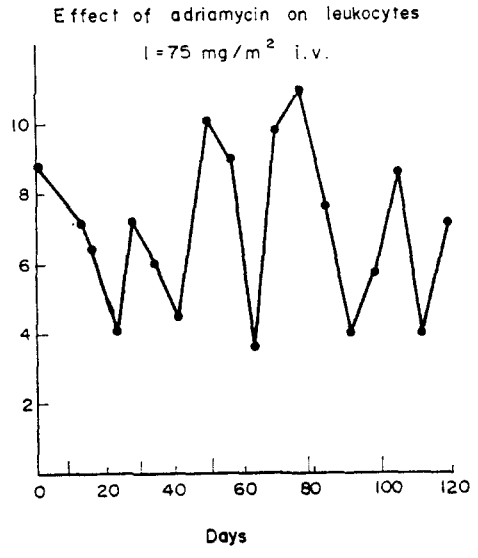

Fig. 2. Effect of 5 doses of adriamycin on the leukocytes. Absence of cumulative toxity.

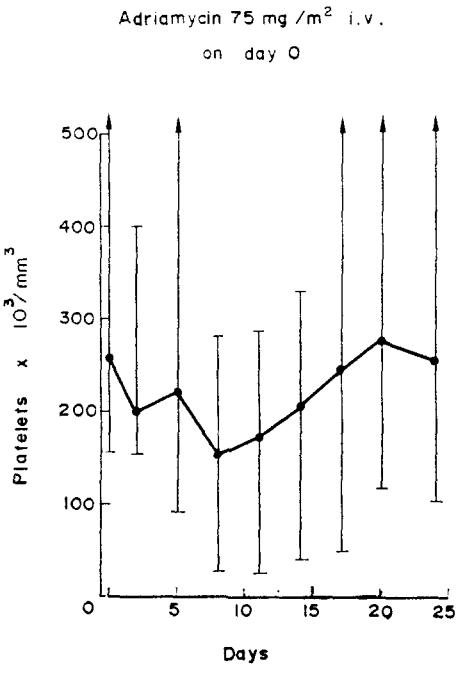

Fig. 4. Median values of the platelets (and range) between 0 and 24 days after one dose of adriamycin.

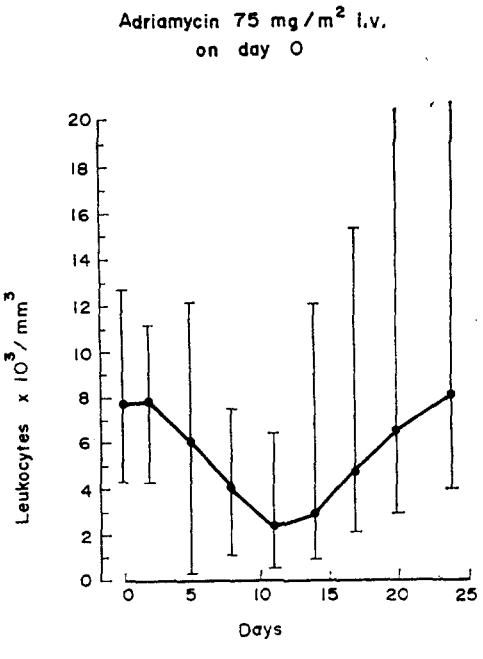

Fig. 3. Median values of the leukocytes (and range) between 0 and 24 days after one dose of adriamycin.

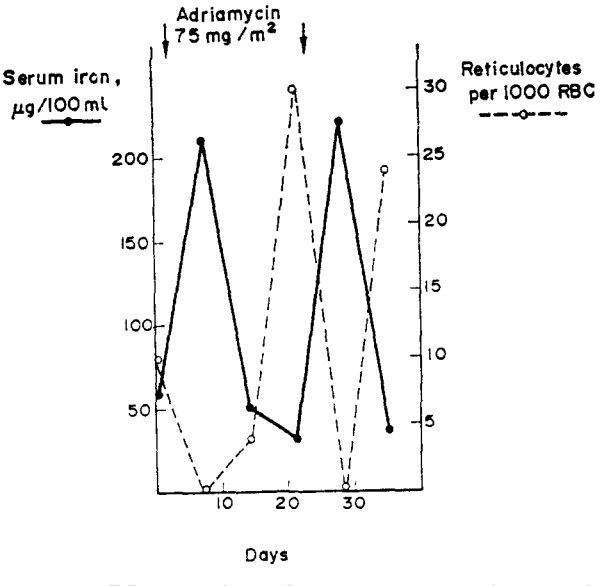

Fig. 5. Effect of 2 doses of adriamycin on reticulocytes and serum iron level.

CONCLUSIONS

This study establishes that an intermittent dose schedule of adriamycin (75 mg/m² i.v. every 3 weeks) is able to induce partial remissions in far-advanced lung cancer. The duration of the remission is not very long and the survival time of the patients does not seem markedly increased.

The median survival of the responders is longer than the median survival of the non-responders (200 vs 162 days), but this difference may be due to the fact that the patients with a longer survival have a greater chance to respond than the poor-risk patients. This seems supported by the observation that the patients who had no prior therapy has a higher proportion of responses and a longer survival than the patients with prior therapy. For the latter, the survival was identical for the responders and the non-responders (Table 3). The evaluation of the antitumour effect is difficult in patients with lung cancer limited to the thoracic cage. A

small decrease of a mass located in a main bronchus may produce the disappearence of atelectasia, and a marked change at the X-ray picture. However, objective effects were observed in well-measurable lesions, such as subcutaneous nodules and lymphadenopathies. With the same criteria of evaluation and on the same type of patients, the Group has observed only one partial remission among 25 cases of epidermoid carcinoma of the lung treated with procarbazine [4] and one partial remission among 11 evaluable cases treated with daunorubicin [5]. By comparing our own results with daunorubicin and adriamycin, this latter drug seems more efficacious in lung cancer, even at a dosage which produces less severe toxicity (Table 4).

Table 4. *Comparison of adriamycin and daunorubicin*

	No. of cases		
	Partial remission	Evaluable cases	Leukocytes < 1·000/mm³
Adriamycin	16	50	4/30
Daunorubicin	1	11	7/11

The 90-day survival of 68% for all cases compares favorably with the results obtained with cyclophosphamide (45·3%), 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (46·7%), and hydroxyurea (approx. 48%), in studies recently published by the VA Lung Cancer Group [6, 7].

The progression of brain metastases during a partial remission of other lesions in 3 cases suggests that adriamycin does not cross the blood-brain barrier in man, as was demonstrated in mice [8].

The interval of 3 weeks between the injections seems adequate: in almost all cases leukocytes and platelets had returned to the normal pre-treatment value after this delay (Figs. 2 and 3). This dose schedule was well tolerated. Cardiac toxicity was very limited and without clinical significance in this group of patients. The most disturbing side-effect was the almost constant alopecia. One particular feature of this regimen is the extremely rare occurrence of stomatitis which was a frequent complication with other schedules. Bonadonna and coll. have observed about 75% of mouth ulcers with their modes of administration [9].

It seems worthwhile trying this intermittent dose schedule in other malignant diseases.

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The Morphology of Phytohaemagglutinin (PHA)-Stimulated Lymphocytes and Permanent Lymphoid Cell Lines Seen by the Scanning Electron Microscope

I. P. GORMLEY and A. ROSS

Medical Research Council, Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland

Abstract—*The morphologies of PHA-stimulated lymphocytes and cells from four permanent lymphoid cell lines were examined with the Stereoscan scanning electron microscope.*

The main features of the cell types observed have been described. The PHA-stimulated lymphocytes showed the characteristic "hand mirror" configuration with well defined uropodia and pseudopodia. Cells of this type were also observed in one of the permanent cell lines. Uropods were not observed in cells of the other three permanent lines and frequently many fine processes occurred either with, or in the absence of, membranous pseudopodia.

INTRODUCTION

THE INTRODUCTION of surface replica techniques and of the scanning electron microscope has greatly facilitated the study of the surface morphology of cells [1–7]. Although the surface structure of human peripheral blood cells has been investigated [8–11] little attention has been paid to the surface morphology of cultured human leukocytes.

Circulating lymphocytes from peripheral blood can be stimulated, by the addition of PHA, to grow and divide [12, 13]. Under "normal" conditions of culture these transformed lymphocytes only survive *in vitro* for short periods before dying. However, lymphoid cell lines can now be established using methods initially described for cell lines derived from patients with Burkitt's lymphoma [14, 15] and more recently from the peripheral blood of patients with a variety of disorders and even from normal individuals [16].

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Lymphoid cell lines are probably derived from some stage in the lymphocytic series as they are known to produce immunoglobulins *in vitro* [17, 18] and have a similar ultrastructure to that described for PHA stimulated lymphocytes [19, 20].

The present report describes the morphology of PHA stimulated lymphocytes and cells from lymphoid cell lines as seen with the scanning electron microscope.

MATERIAL AND METHODS

Lymphocytes, containing not more than 1% granulocytes, were separated from human peripheral blood by gradient centrifugation [21] and resuspended in Ham's F10 medium [22] supplemented with 20% foetal calf serum and 10% tryptose phosphate broth. Five ml. cultures were set up at $2–4 \times 10^6$ lymphocytes/culture and phytohaemagglutinin (Wellcome MR10) was added to give a final concentration

of 0·05 μgm/ml. The cultures were then incubated for 72 hr at 37°C.

Four lymphoid cell lines were examined: EB2 [15] and JIJOYE [23], both of which were derived from patients with Burkitt's lymphoma, FLE_1 [24] derived from a patient with infectious mononucleosis, and CLA_2 [25] derived from a sample of placental blood by a cocultivation technique. All lines were routinely grown in Ham's F10 medium supplemented with 20% foetal calf serum and 10% tryptose phosphate broth.

Cells were fixed for scanning electron microscopy by adding an equal volume of 5% glutaraldehyde in 0·2 M cacodylate buffer directly to the culture medium at 37°C and left for 30 min. The cells were gently washed four times in 0·2 M ammonium acetate by low speed centrifugation (*circa.* 30**g**) and drops of the final suspension were allowed to dry at room temperature on a clean coverslip in a dust free atmosphere. Portions of the coverslips were attached to specimen holders and vacuum coated with a layer of gold/palladium. The cells were examined and photographed using a Mark IIA Cambridge Stereoscan Electron Microscope.

RESULTS

The majority of PHA-stimulated lymphocytes seen with the scanning electron microscope [Fig. (1a), (b)] have a "pear" shaped body which narrows posteriorly forming a tail or uropod. The uropod thickens towards the tip and frequently small processes can be seen extending from this thickened area. A slightly ruffled membranous pseudopod of variable size often appears around the thickest part of the body of the cell. A typical transformed lymphocyte is shown in Fig. 1(a) with the pseudopod extending right round the anterior end of the cell and Fig. 1(b) shows another cell in which the pseudopod is much smaller and which has formed an attachment to a neighbouring cell resulting in an irregular shape to the body of the cell.

In only one of the four lymphoid cell lines examined, CLA_2, were cells resembling PHA-stimulated lymphocytes observed. Figure 1(c), (d) show two examples of such cells with pear shaped bodies, uropodia and pseudopodia. Other forms were also found as can be seen from the surrounding cells in Fig. 1(c), (d) and from the two cells shown in Fig. 1(e) in which no obvious pseudopod or uropod can be seen.

The most striking feature about the other three lines, EB2, JIJOYE and FLE_1 was that on only one occasion was a cell observed to have a structure resembling a uropod [Fig. 1(f)]. Pseudopodia, however, resembling those of PHA-stimulated lymphocytes were frequently found in all four lines. Figures 2(a)–(d) show various examples of the types of cell observed. One or more pseudopodia were observed and frequently many small processes extended out from other parts of the cell.

A further type of cell occurring in all four cell lines had no pseudopod or uropod but many fine processes were observed either radiating out from the periphery of the cell [Fig. 1(e)] or mainly confined to one end [Fig. 1(f)].

Lymphoid cells often form small clumps in culture and processes similar to those described above were commonly seen extending from the periphery of these clumps.

DISCUSSION

The appearance of the transformed lymphocyte as observed using the stereoscan microscope was in good agreement with previous light [26–29] and electron microscope [30] observations. The lymphocytes often assumed a characteristic "hand mirror" configuration with a pseudopod of variable size anteriorly and a very prominent uropod posteriorly. Such forms have been described for motile lymphocytes *in vitro* [26, 27], PHA-stimulated lymphocytes [29, 30] and as a characteristic feature of cells in mixed lymphocyte cultures [28].

Several authors [17–20] have suggested that, on ultrastructural and immunological grounds, there was no difference between phytomitogen-stimulated lymphocytes and those of established cell lines. At least in the line CLA_2, our observations suggest that the morphology of some cells from permanent lymphoid lines can also be very similar to that of transformed lymphocytes. However the cells of permanent cell lines were very variable in their morphology both within a given cell line and between cell lines. Furthermore in JIJOYE, EB2 and FLE_1, no cell was observed which showed the typical transformed lymphocyte morphology. This was often due to the apparent loss of any structure resembling a uropod, although many cells, in all the cell lines examined, had a pseudopod which appeared similar to that observed in transformed lymphocytes in that it was frequently membranous and slightly ruffled.

Time-lapse micrographic observations [26–28] have shown that not all stimulated lymphocytes show uropodia as this specialised state

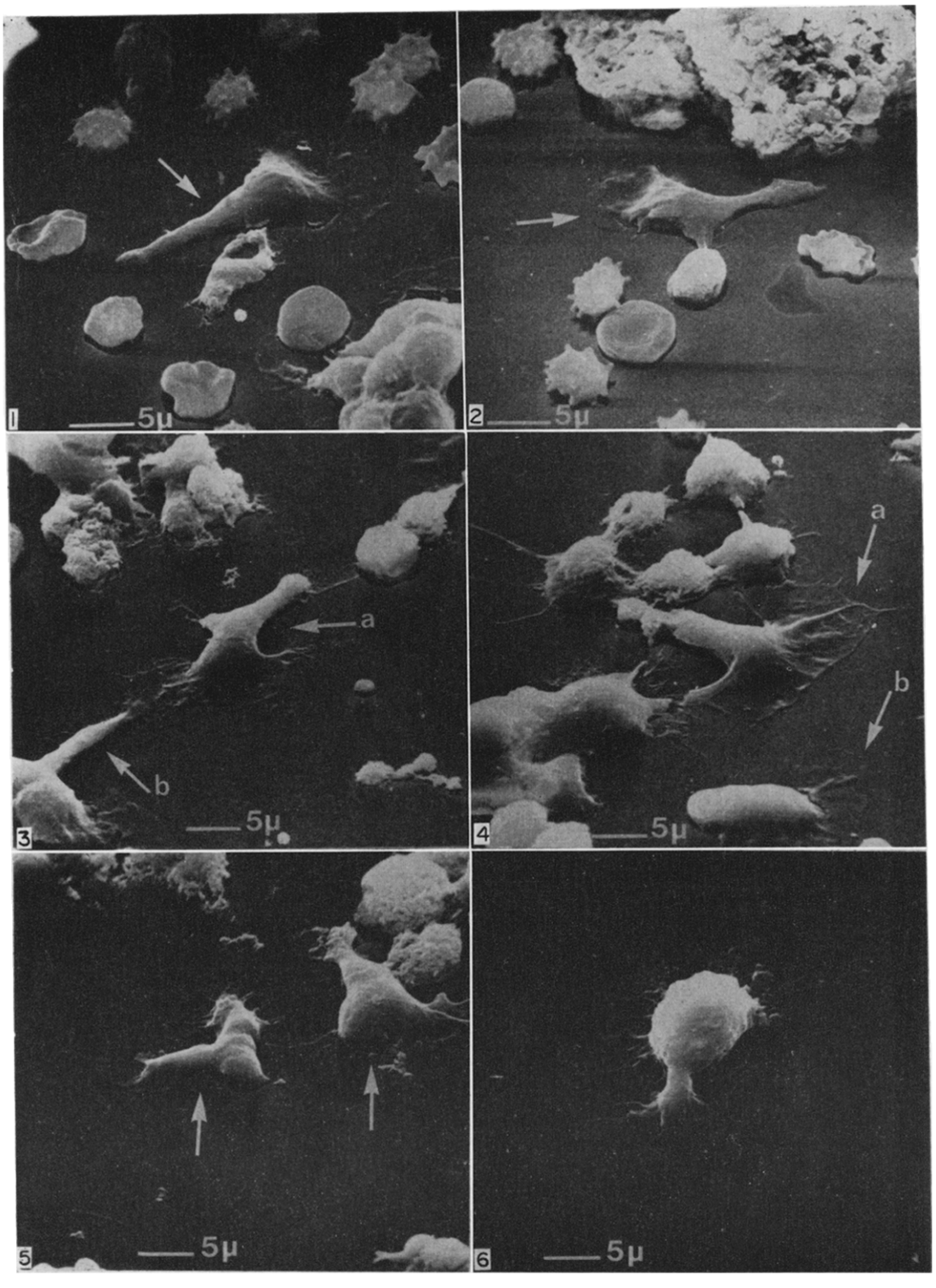

Fig. 1

(*a*) *Lymphocyte stimulated for* 72 *hr with PHA. The cell* (*arrowed*) *shows the characteristic "hand mirror" configuration.* (*The other cells seen in this micrograph are erythrocytes and a clump of lymphocytes*).

(*b*) *Lymphocyte stimulated with PHA for* 72 *hr. This cell shows a distorted "hand mirror" form due to a process formed on the side of the cell.*

(*c*) *CLA_2 cell showing a "hand mirror" configuration with well defined pseudopod and uropod* (*arrowed* a). *A further uropod can be seen extending from a clump of cells at the bottom left hand corner of the micrograph* (*arrowed* b).

(*d*) *CLA_2 cell again showing a "hand mirror" configuration* (*arrowed* a). *Other types of process can be seen extending from the other cells in the micrograph. A cell at the bottom shows a pseudopod but no uropod* (*arrowed* b).

(*e*) *Two CLA_2 cells without easily recognisable pseudopodia or uropodia* (*arrowed*).

(*f*) *EB2 cell showing a short uropod-like structure but no pseudopod.*

(to face p. 492)

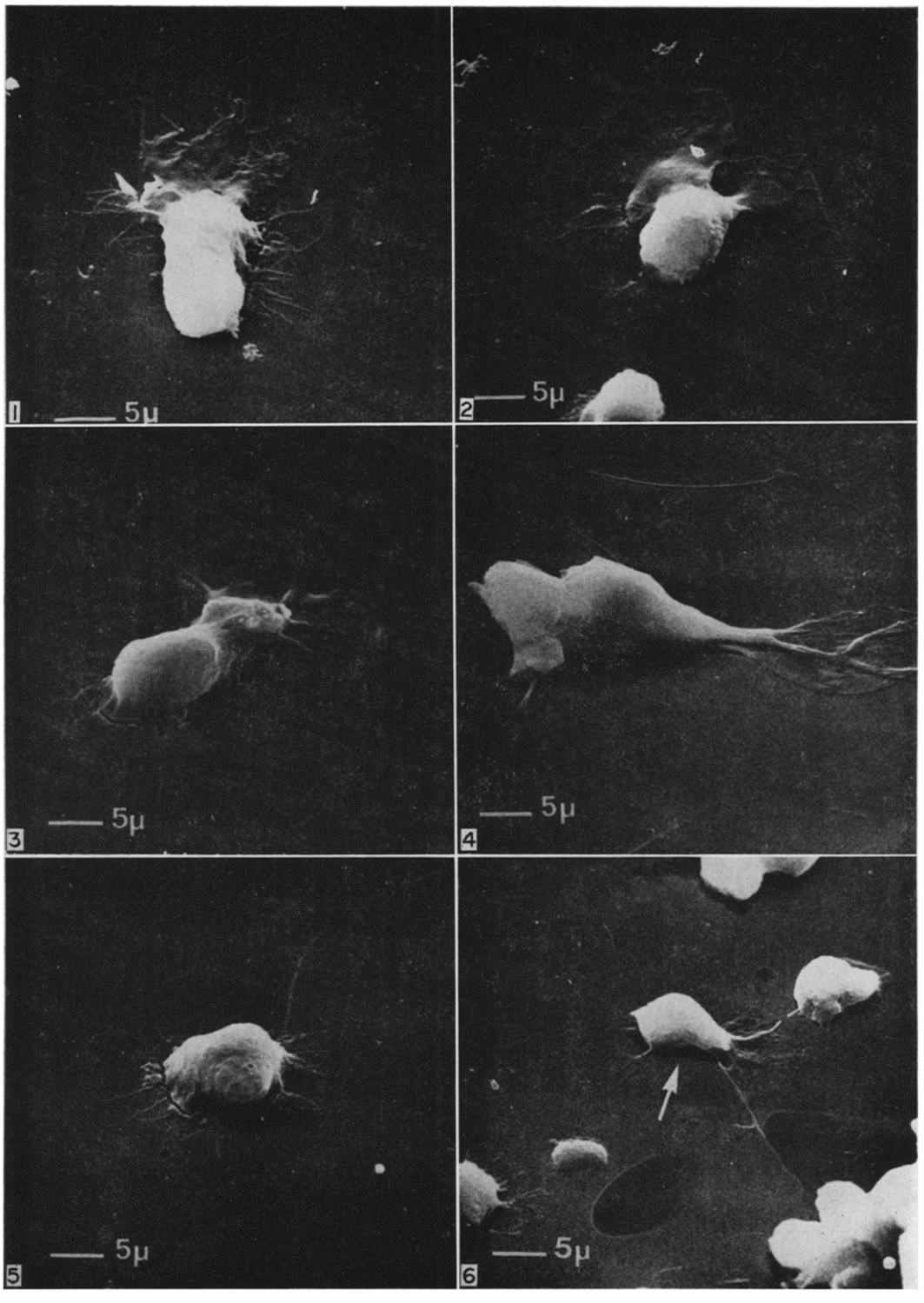

Fig. 2
(*a*) *EB2 cell with a ragged pseudopod and multiple spike-like processes.*
(*b*) *JIJOYE cell showing two independent pseudopods.*
(*c*) *EB2 cell with two opposing pseudopods.*
(*d*) *JIJOYE cell showing a strangely-shaped pseudopod.*
(*e*) *EB2 cell with multiple spike-like processes radiating round the periphery of the cell.*
(*f*) *FLE_1 cell with multiple spike-like processes extending from one side of the cell (arrowed).*

is reversible. It is possible therefore that the cells in lymphoblast lines have lost the ability to form uropods to varying degrees in different lines.

All the forms observed by Clarkson *et al.* [31] in a study of the morphology of permanent lymphoid cell lines derived from patients with acute leukaemia were seen in the three lines JIJOYE, EB2 and FLE_1, maintained in our laboratory, all of which had a similar morphology. However, the morphology of cells from the CLA_2 line was surprising as they more closely resembled PHA-stimulated lymphocytes than other reported lymphoblastoid cells.

The small number of cell lines that we have been able to observe precludes any correlation of morphology with regard to the origin, time in culture, or karyotype of the cell lines. A survey of a much larger number of cell lines could well reveal such correlations.

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Spontaneous Immunity to Mammary-Tumour Virus (MTV)-Associated Antigens in Mice and its Influence on Syngeneic Mammary-Tumour Growth

M. MÜLLER and ST. ZOTTER

Medical Academy "Carl Gustav Carus", Institute of Pathology, Dresden, Germany (GDR)

Abstract—*Peritoneal cells from mice untreated but naturally infected with MTV (CBA/Bln mice and (XVII ♀ × CBA ♂) F_1-hybrids) showed inhibited migration when incubated with MTV-containing tumour preparations. Incubation of those cells with MTV-free tumour or tissue preparations had no influence on the migration. Peritoneal cells from MTV-free XVII/Bln mice were inhibited neither by MTV-containing tumour preparations nor by MTV-free materials. These findings may indicate that mice naturally infected with MTV can develop some degree of cell-bound immunity to MTV-associated antigens without any further antigenic stimulation. This phenomenon is in line with the previous detection of serum antibodies to MTV in untreated but infected mice of several strains. Three grafted mammary tumours sharing MTV-associated antigenicity grew faster in syngeneic CBA mice with spontaneous antibodies to MTV than in mice without detectable antibodies. On the contrary, the results of macrophage migration inhibition tests obtained for every single host before tumour challenge did by no means correlate with the tumour growth. The possibility is discussed that spontaneously occurring antibodies to MTV may facilitate mammary tumour growth. The missing correlation between MTV-dependent macrophage migration inhibition and tumour growth may be due partially, at least, to an interference of the spontaneous humoral response to MTV-associated antigens with the cell-bound one.*

INTRODUCTION

TRANSPLANTATION experiments led to the assumption that mice naturally infected with MTV are truly tolerant to antigens associated with MTV [1–5]. The tolerance concept, however, was disproved by experimental results which showed that antibody response to MTV can be induced in mouse strains infected with MTV [6]. Such antibodies were also demonstrated in non-treated but autochthonous MT bearing C3H mice [7]. Recently Müller *et al.* [8] have shown that the spontaneous occurrence of antibodies precipitating the whole virion or an antigen of the outer viral coat is a more frequent finding in the sera of multiparous female mice. These antibodies were found both in mice infected with milkborne MTV (C3H, CBA) and with eggborne MTV (GR, C3Hf). It appears possible that the lactation-dependent release of virions into the blood accounts for the endogenous immunization. This phenomenon of spontaneously occuring immunity was called "underground immunity" [9].

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Abbreviations:
MT(s) = mammary tumour(s)
BSA = bovine serum albumin
MMI = macrophage migration inhibition

The role played by this type of immunity in development and growth of mammary tumours is not clear. Furthermore the question arises as to whether or not there exists also a cell-bound underground immunity in MTV-infected mice. This report deals with experimental results indicating the existence of delayed hypersensitivity to MTV-associated antigens in untreated but infected mice and showing the humoral underground immunity to cause enhancement of syngeneic MT growth rather than inhibition.

MATERIAL AND METHODS

Mice

CBA/Bln mice were used which are maintained continuously by single line brother × sister mating in our laboratory. More than 90% of the CBA females have immunologically detectable amounts of MTV in their milks during the first lactation. Strain XVII/Bln mice, also used in this trial, never develop MTs and are free of any MTV antigenicity in milk and tissues [10].

(XVII ♀ × CBA ♂) F_1 hybrids are infected, obviously by the male parents [10].

Tumors

MTs of CBA female origin serially grafted in that strain were used. MT4814, MT6415, and MT8020 produce continuously large amounts of A- and B-particles and are rich in MTV-antigens [11]. They share cross-antigenicity in transplantation experiments and in colony inhibition tests [10]. MT9915 is free of fluorescent-immunologically detectable amounts of MTV antigens, contains extremely rare electron microscopically visible MTV-particles but shows cross-antigenicity with MT4814 and MT8020 in transplantation assays and colony inhibition tests. MT7782 is a primary MT spontaneously transformed into a sarcoma during the 6th syngeneic passage and now totally free of MTV-particles, MTV-antigenicity, and cross-antigenicity with the three other tumors.

MMI

Mice were injected intraperitoneally with 0·2 ml sterile mineral oil 48 hr before washing out the peritoneal cells with medium (Eagle MEM with 10% inactivated calf serum obtained from "Staatliches Institut für Immunpräparate und Nährmedien", Berlin, GDR). The medium contained 100 E/ml of penicillin and 100 μg/ml of streptomycin in all steps of the test. After twofold washing with medium, cell suspensions were counted and adjusted to 8×10^6 cells/ml. As to be described, cells were harvested from pooled mice in Experiment 1 and from single mice in Experiment 2. Peritoneal cell suspension was filled into sterile capillary tubes closed at the bottom with Mikropal [12] and centrifuged at 500 rev/min for 5 min. Every tube contained 3×10^5 cells. The tubes were cut at the cell-fluid interface. The ends of the tubes containing the pellet were incubated in a chamber containing 1 ml medium with 10% of the respective antigen solution. The antigen content was heightened to 20 or 50% in some assays. After an incubation period of 24 hr at 37°C the areas of cell migration were projected on paper and measured by planimetry. Inhibition was recorded in percent of migration as compared with the same cells incubated without antigen.

Migration index (MI) =

Migration area after incubation with antigen (mm^2)/Migration area after incubation without antigen (mm^2)

The significance of inhibition was calculated by the "Zeichentest" [13], comparing every single value of the test incubations with the respective value of control incubations according to the range order.

Antigen

Transplanted MT 4814 was homogenized at 4°C for 2 minutes at 14,000 rev/min. After centrifugation at 6,000 rev/min for 10 min the supernatant was centrifuged at 198,000 **g** for 1 hr and the resulting pellet re-suspended in medium.

The suspension was centrifuged at 6,000 rev/min for 10 min and the supernatant containing the MTV-particles was adjusted to a protein content of 1 mg/ml. The antigen preparation was thus ready for MMI tests. The preparations were stored in ampoules at −20°C. The MTV-free MT 7782 was prepared by the same procedure. Whole homogenate of foetuses of strain XVII (18th day of pregnancy) cleared at 6,000 rev/min and adjusted to 1 mg protein/ml served also as MTV-free control. A further MTV-free control was XVII female serum mixed with 10% BSA and adjusted to 1 mg protein/ml.

Immundiffusion

The micro Ouchterlony test [14] applied for MTV by Fink *et al.* [15] was used to test mouse sera against purified B-particles. MTV-infection

of mice was tested using stomach milk treated with Versene (0·025 m in tris HCl buffer; at pH 7·4) and ether. Specific rabbit anti MTV-sera were allowed to react with the milk [11]. The B-particles were prepared using the technique of Calafat and Hageman [16]. The rabbit anti MTV-sera produced in our laboratory had the same specificity when compared with a serum kindly provided by Daams [17].

In vivo experiments

Tumour cell suspensions were prepared as described earlier [18]. Challenge inocula were given subcutaneously in 0·1 ml volumes containing predetermined doses.

CBA mice were inhibited by these antigens regardless of whether they were obtained from immunized animals or from normal mice. Therefore we started to test the working hypothesis that normal CBA mice and their F_1-hybrids develop some degree of underground hypersensitivity as a result of their natural MTV infection.

As shown in Table 1 peritoneal cells from normal CBA males were inhibited after incubation with MTV-containing preparations from MT 4814. Tissue preparations from foetal XVII mice and XVII strain serum mixed with BSA did not inhibit migration at all. Peritoneal cells from MTV infected female (XVII ♀ ×

Table 1. *Macrophage migration of nontreated mice*

Mice	Migration index (MI)*					
	Antigen MT4814 (MTV+)			Antigen XVII fetal tissue (MTV−)		Antigen XVII ♀ serum + BSA (MTV−)
	10%	20%	50%	10%	50%	20%
CBA ♂†	0·76‡	0·61	0·35	0·91	0·87	1·01
XVII ♂†	1·20	1·07	1·22	0·98	2·50	0·92

	Antigen MT4814 (MTV+)	Antigen MT7782 (MTV−)
	20%	20%
XVII ♀ × CBA ♂ F_1 ♂†	0·70	1·05

$$*MI = \frac{\text{Migration with antigen (mm}^2\text{)}}{\text{Migration without antigen (mm}^2\text{)}}.$$

†Peritoneal cells pooled from 6 mice.

‡Mean indices from 8 capillary tubes. The indices underlined represent significant ($p < 0·05$) inhibition.

RESULTS

Experiment 1

In a series of preliminary experiments we tried to induce delayed hypersensitivity in both male and female XVII and CBA mice. Delayed hypersensitivity can be investigated *in vitro* by means of the MMI test [19]. Macrophages of mice immunized with MTV-containing tumour preparations or purified B-particles with or without complete Freund's adjuvant failed to give migration indices lower than 0·85 after incubation with the respective antigen. Whereas peritoneal cells of normal or immunized XVII mice migrated without any inhibition effect in the presence of MTV preparations, cells of CBA ♂) F_1-hybrids showed inhibited migration when incubated with MT4814 antigen but did not do so after incubation with antigen from MTV-free MT7782.

Experiment 2

Twenty-nine CBA virgin females were mated with CBA males and thereafter maintained in single cages. Each litter was killed on the third day and the stomach milk was pooled to test the MTV- infection of the respective mother. All 29 females had precipitable amounts of MTV in their milk. Ten days after removing the litters the mice were bled from the retro-obital sinus and the 29 sera were frozen at −20°C until use in micro Ouchterlony test against MTV. Subse-

quently the peritoneum of each single mouse was washed with medium under ether anaesthesia and the peritoneal cells were tested for MMI with 10% of a standard preparation of MT4814 containing abundant B-particles in medium frozen at −20°C till use.

Summarizing the findings the following relationships between the parameters measured can be described (Fig. 1): 15 out of 29 mice were designated to show "enhanced" tumour growth. Ten of these 15 mice had spontaneous antibodies precipitating the whole virion in

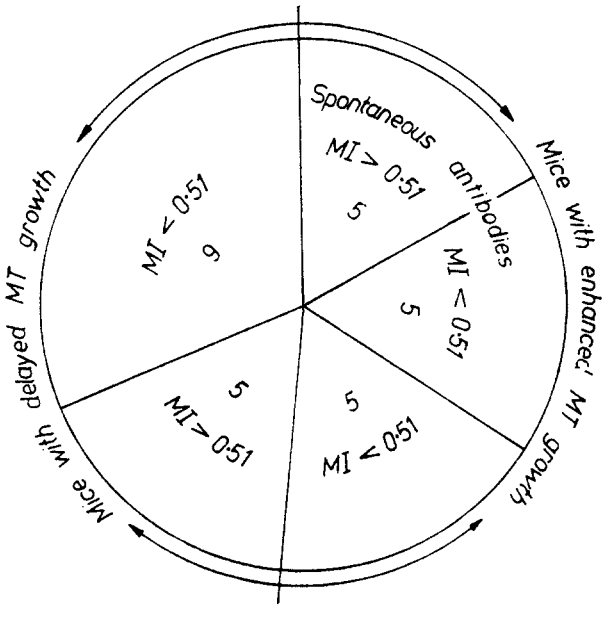

Fig. 1. Diagram showing the distribution of spontaneous antibodies to MTV and the migration indices (MI) among mice with "enhanced" (right half) and "delayed" (left half) tumour growth. The number of mice is listed in the respective sector.

The cell number obtained allowed only for incubate of two tubes per mouse, one with antigen and one without it. MMI was considered to be real only if the MI was lower than 0·51. On the next day, the mice were injected with challenge inocula of MTs sharing MTV-associated antigenicity as had been tested before. Ten mice received 4×10^4 MT 8020/9 cells, 10 mice received 4×10^4 MT 9915/1 cells, and 9 mice were injected with 10^5 MT 6415/5 cells. (The number behind the oblique line expresses the transplantation generation of the MT). When 8 mice in each tumour group had developed palpable tumours, the mice were killed and the tumours were weighed. Thereafter each group was subdivided into two halfs, one half with the larger tumours and the other half with the smaller tumours including the mice without tumour takes. The first half was called "enhanced tumours" and the second was called "delayed tumours". The group of 9 mice challenged with MT6415 was arbitrarily subdivided into 5 "enhanced" and 4 "delayed" tumours.

agar. Five mice were without detectable antibody activity, but had signs of delayed hypersensitivity to MTV-associated antigens. In addition 5 mice with antibodies also gave an MI indicating delayed hypersensitivity. Thus it can be assumed that in the case of "enhanced" tumour growth the only important factor so far detectable is the occurrence of spontaneous antibodies to MTV. This assumption is supported by the fact, that not 1 out of the 14 mice with "delayed tumour growth" had detectable antibodies. The hypothesis of MT enhancing activity of the spontaneous antibodies seems to be valid in our experimental system, since the finding of antibodies in 10/15 mice with "enhanced" MT growth and in 0/14 mice with "delayed" tumour growth is significant ($\chi^2 = 6{\cdot}62$ $p < 0{\cdot}01$). The peritoneal cells from 9 out of 14 mice with delayed tumour growth were inhibited in the presence of MTV. On the basis of statistical analysis (χ^2-test) this phenomenon has to be considered without influence on the growth of immunogenic mammary tumours in our system.

DISCUSSION

Our findings show that migration of peritoneal cells of normal male and female CBA mice and their F_1-hybrids infected with MTV is inhibited in the presence of MTV antigens and might support the hypothesis that there also exists some degree of cell-bound underground immunity similar to MTV as was demonstrated for humoral immunity [8]. The specificity of the MMI observed is underlined by the fact that cells from uninfected XVII mice were not inhibited by MTV-associated antigens and that MTV-free tissue preparations evoked no inhibition effect on the cells of either strain. But the possibility cannot be ruled out entirely that the inhibition effect may be due rather to a more direct action of MTV on macrophages, possibly particularly sensitive in CBA mice, than to delayed hypersensitivity.

Transplantation immunogenicity of MTV-induced MTs is, at least, partially dependent upon MTV-associated antigens [20]. Contrary to our findings, one could expect the MTV-evoked MMI to correlate with the delay of MT growth. But we feel that the interference between humoral and cellular immune factors may be of most importance in the system employed. The finding of spontaneous antibodies against MTV in two thirds (10/15) of mice with enhanced tumour growth fits well with the hypothesis that these spontaneous antibodies may facilitate MT growth in the sense of immunological enhancement. It seems likely that the number of mice with a low titer of spontaneous antibodies is much greater than can be revealed by the micro Ouchterlony technique. The enhancing effect of these antibodies, however, may interfere with the cellular factors of underground immunity.

This may explain the missed correlation between MMI and delayed tumour growth. On the other hand it seems possible that MMI actually reflects a state of delayed hypersensitivity to MTV, but that the cytotoxic capability of immune lymphocytes responsible for delayed tumour growth *in vivo* is not expressed by MMI test. Some other findings indicate that the so-called underground immunity may play a role in the development and growth of MTs. After neonatal thymectomy MT incidence in infected mice is delayed and/or reduced [21–23]. One may assume like Heppner [24] that thymectomy interferes with the production of antibodies to MTV-associated antigens which can enhance tumour growth when they are produced. Heppner [24] showed the appearance of antibodies capable of abrogating the inhibitory effect of lymph node cells to MT cells *in vitro* to be suppressed, indeed, after thymectomy. But in Heppner's experiments the antigens involved seem to be not MTV-associated but individually specific. Yunis *et al.* [22] found that spleen cells of adult uninfected mice restored the MT incidence to normal levels after thymectomy and spleen cells from MTV-infected mice did not do so. Attempts were made to explain this phenomenon in two different ways. Heppner [24] discussed the theory that only spleen cells from MTV-free mice are not tolerant and can transfer the capability of producing antibodies enhancing tumour growth. Bentvelzen [7], however, thought that the spleen cells of infected mice transfer some degree of underground immunity. A third explanation could be that thymectomy abrogates the capability to develop both cellular and humoral underground immunity and that spleen cells from infected mice would transfer predominantly cell-bound underground immunity whereas spleen cells from MTV-free mice would be primed to produce enhancing antibodies in the infected hosts. Sublethal *x*-irradiation before tumour grafting results in a delay of syngeneic MT-growth in infected mice but did not so in MTV-free mice [25]. On the basis of the findings discussed here, we would like to assume that *x*-irradiation resulted predominantly in a suppression of the antibody response to MTV-associated antigens thus preventing immunological enhancement.

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An Attempt at Selective Chemotherapy of Hypoxic Cells: Triethylenemelamine and Irradiation of a C3H Mouse Mammary Tumour

I. F. TANNOCK*†, NANCY MARSHALL* and L. M. VAN PUTTEN‡

*Section of Experimental Radiotherapy, University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77025 U.S.A. ‡Radiobiological Institute TNO, Lange Kleiweg 151, Rijswijk Z.H., The Netherlands

Abstract—*Hypoxic cells are resistant to radiation and, because they are predominantly non-cycling, to many antimetabolites. However, production of lactic acid by hypoxic cells in tumours probably leads to a rather low pH in the surrounding extracellular fluid; such cells might therefore be sensitive to cytotoxic drugs which are either selectively activated or selectively deposited under acidic conditions. We have used one such drug, the alkylating agent triethylenemelamine (TEM), with irradiation of a transplanted C3H mouse mammary tumour. Injection of TEM had no significant effect on the mean total dose of radiation (given either as a single dose or as 10 equal fractions) required to obtain local control of tumours in 50% of animals; however, the drug caused a significant prolongation in the time to tumour regrowth. Administration of glucose in the drinking water of the animals did not increase the effectiveness of tumour irradiation, or potentiate the action of TEM. Injection of TEM also had no influence on the time of appearance or severity of skin reactions after irradiation. The effect of TEM on proliferating and non-proliferating bone marrow stem cells was assayed by the spleen colony method: it was found that non-proliferating cells were more resistant to the drug. Thus, the resistance of hypoxic tumour cells to the lethal action of the drug might be due partly to their non-cycling state.*

INTRODUCTION

WHEN radiotherapy fails to result in local control of tumours, it is probably the cells that are hypoxic at the time of irradiation which survive and regenerate the tumour. Thus, if supportive chemotherapy is to be effective, it should be aimed specifically at sterilizing hypoxic, radioresistant cells. However, hypoxic cells appear to be predominantly non-proliferating [1–4] and are therefore resistant to many antimetabolites. Hypoxic cells are also situated distant from functional capillaries and are often close to regions of necrosis; thus diffusion of drugs that are rapidly metabolized results in lower concentrations in hypoxic tumour regions than in the vicinity of cells near blood vessels. Also agents which are analogues of normal metabolites may compete with a large endogenous pool diffusing from regions of necrosis; the latter effect is suggested by observation of low grain counts over the few cells near necrotic tumour regions which are labelled after injection of tritiated thymidine [2, 4]. The hypoxic cell may therefore be resistant to many types of chemotherapy as well as to radiotherapy.

An important property of the hypoxic cell is its dependence on anaerobic glycolysis for a source of energy. Tumours in general are more acidic than normal tissues [5, 6] and production of lactic acid by hypoxic cells probably leads to a particularly low pH in their immediate environment. This property might be utilized in planning chemotherapy to attack selectively the hypoxic cells. Certain aromatic nitrogen mustards tend to be deposited in regions of

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†Present address: Dept. of Radiology, Hospital of the University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

low pH, because fast diffusing or relatively insoluble ionic forms may then predominate [7, 8]. Other drugs have greater activity at low pH: these include 5-fluorouracil [9], tetra-azatricyclododecane [10] and triethylenemelamine (TEM); the last is an alkylating agent which has a high value of the acid catalysis coefficient [11]. Infusion of glucose into tumour-bearing animals has been found to cause a large decrease in mean tumour pH with little effect on the pH of normal tissues [6]; this procedure was able to potentiate the action of each of the above drugs [9, 10, 12].

In the present study a C3H mouse mammary tumour that is known to contain hypoxic cells [13] has been irradiated with single or fractionated doses, while host animals have been injected with TEM or saline. Damage to normal tissues by radiation in animals injected with TEM or saline was assayed using the endpoints of moist desquamation and epilation of the skin. We also studied the effect of pre-administration of glucose in the drinking water. Administration of glucose might reduce oxygen consumption through the Crabtree effect, and, by allowing oxygen to diffuse to previously hypoxic cells, might modify the radiation response [14]; it might also potentiate the action of TEM [12].

The drug TEM was chosen for this initial study because its previous clinical use with irradiation of a few types of tumour had suggested some therapeutically beneficial effects [15, 16]. Also, most of the hypoxic cells in the present tumour have been shown to be non-proliferating [4]: while almost all drugs (including TEM) are more lethal for proliferating than for non-proliferating cells [17-19], we thought that differences in sensitivity should be less for an alkylating agent like TEM than for an antimetabolite. In an additional experiment we have used the spleen colony assay to assess the effect of TEM on proliferating and non-proliferating bone marrow stem cells.

MATERIALS AND METHODS

C3H/He mice were used in toxicity studies and in assays of tumour and skin response to radiation. Mice were maintained under SPF conditions and were 8 to 12 weeks old at the start of each experiment. Conventionally-maintained F_1 hybrid mice (C57BL/Rij × CBA/Rij) were used in spleen colony assays.

TEM was supplied by Lederle Laboratories, New York, and was diluted in physiological saline shortly before injection. In initial studies of toxicity, groups of about 7 mice were given graded doses of the drug as a single i.p. injection, or as 5 or 10 consecutive daily doses. Body weights were recorded each day and the LD50 values were estimated. The consumption of graded concentrations of glucose (0 to 10%) in the drinking water, and its effect on mouse body weight, were also measured.

Assays of radiation response

The tumour used in the present studies was a fourth generation isotransplant of a spontaneous C3H mouse mammary adenocarcinoma. To generate tumours, a cell suspension was prepared by a method described previously [13] and 10 μl volumes containing about 10^5 living cells were injected into the right legs of recipient mice. Tumours were irradiated when they attained a mean diameter of 8 to 9 mm, usually about 2 weeks after transplantation. Mice were anaesthetized with nembutal, and taped to a rectangular brass plate, with the tumour positioned centrally over a 3 cm diameter aperture. For irradiation of skin the same procedure was used except that the normal right leg was placed over the aperture. The plate was then placed in a brass chamber, and the latter was rotated within a Cs^{137} irradiator employing twin opposed sources until the aperture and tumour (or normal leg) were aligned in the radiation field. The dose rate was about 1050 rad/min, and variation of dose over the field was less than 5% of the mean.

The radiation response of tumours was assayed using the endpoint of local tumour control for a specific time period. About 40 to 50 tumours were implanted for each assay, and groups of 4 to 6 mice received graded doses of radiation to their tumours. Animals were checked for tumour recurrence at weekly intervals up to 130 days after irradiation; previous experience has shown that later recurrences are rare. A regrowing tumour was scored as a definite recurrence when it attained the same mean size as at irradiation. After lower doses most of the tumours recurred quickly in the radiation field, while after higher doses they recurred after a later interval or not at all. Logit regression lines were computed to the relation between probability of tumour control for a specified time (x days) after irradiation and radiation dose [20], and the endpoint used was the tumour-control-dose–50% (TCD–50/x) defined as:

TCD–50/x = Mean radiation dose yielding local tumour control in 50% of the animals for x days.

Estimates of TCD–50/x and its 95% confidence

limits were computed for each assay, and for x = 60, 80, 100, 130 days; estimates of the p-value for 2 groups of mice (e.g. those injected with TEM or saline) were based on Student's t-test. A comparison of values of TCD–50 for x = 130 days gives information on the ability of treatment to influence permanent local control; the same comparison for smaller values of x gives information on regrowth delay.

To test the effect of glucose administration and TEM injections on tumour response to a single dose of radiation, about 160 animals were implanted with tumours and were randomly assigned to one of four equal treatment groups:

(1) mice given 10% glucose in the drinking water and injected with TEM;

(2) mice given 10% glucose in the drinking water and injected with saline;

(3) mice given water to drink and injected with TEM;

(4) mice given water to drink and injected with saline.

Mice were separated into groups drinking water or glucose when their tumours had a mean diameter of 6·5 mm; they were irradiated 3 days later (when the mean tumour diameter averaged 8 to 9 mm) and all animals drank water after irradiation. TEM was injected i.p. at a dose of 1 mg/kg body weight on the evening before the day of irradiation, and on the morning before and evening after irradiation; controls received injections of saline. Scoring of recurrences was performed after coding the animals, in order to prevent subjective bias.

We also studied the effect of TEM injections on the response of tumours to 10 equal daily fractions of radiation. TEM was injected i.p. at a dose of 0·5 mg/kg on the morning before each fraction and controls were injected with saline. Animals were not given glucose in this experiment. Values of TCD–50 were evaluated in terms of total radiation dose to give local control of tumours.

The influence of TEM injections on the response of normal mouse skin to irradiation with a single dose or with 10 equal fractions was assayed using the endpoints of moist desquamation or epilation. TEM or saline were injected at the same dosage and times as in the experiments with tumours. Groups of about 40 mice per assay were used and sub-groups of 4 to 6 animals received graded doses of radiation to their right legs. The legs were inspected at intervals from 19 to 34 days after irradiation (after the first dose, when 10 fractions were given); reactions were scored using a zero to four-plus system and the treatment history of the animals was unknown to the observer. Results were analyzed in a similar way to those for tumour recurrence: we calculated the mean doses of radiation to give moist desquamation or complete epilation within the irradiation areas of 50% of the animals at specified times after irradiation.

Spleen colony assays

The spleen colony assay of Till and McCulloch [22] was used with minor modifications to study the effect of TEM on essentially non-proliferating colony-forming units (CFU) of mouse bone marrow. Technical details have been published elsewhere [17]. Briefly, mice were injected s.c. with single doses of TEM ranging from 0 to 1·25 mg/kg, and were killed 16 hr later. Suitable dilutions of bone marrow cell suspensions were prepared from the femurs of these mice, and were injected into the tail veins of isologous mice that had been irradiated with 750 rad 1 to 2 hr earlier. Nine days later, spleen colonies with diameters of 0·25 mm or more were counted with a dissecting microscope.

A variation of the above method was used to assay the effect of TEM on proliferating CFU [17]. Mice were lethally irradiated (750 rad) and were injected with a standard amount of isologous bone marrow. After 7 days the spleens of such chimeras contained exponentially-increasing numbers of CFU. At this time, the mice were injected with TEM at a range of dose levels, and were killed 16 hr later. Cell suspensions were made from their spleens, and the number of surviving CFU was assayed in new mice by the spleen colony method described above. Results were expressed as percentages of CFU per spleen compared with untreated controls assayed simultaneously.

RESULTS

Toxicity experiments

Results of toxicity studies with TEM are summarized in Table 1. Dose response curves are rather steep: thus the schedule of 10 daily injections of 0·5 mg/kg TEM used in experiments with fractionated irradiation caused 15 to 20% loss of body weight, but no deaths among about 90 mice thus treated, while 1 mg/kg/day was 100% lethal. All mice recovered their weight loss rapidly when the injections were stopped.

Administration of glucose in the drinking water at concentrations of 0 to 10% had no demonstrable toxic effects on the animals.

Mice given higher concentrations of glucose drank more and gained up to 10% in body weight compared with controls.

Table 1. *Results of toxicity experiments for injection of TEM in C3H/He mice*

Total dose (mg/kg)	Fraction of survivors after:		
	Single injection	5 daily injections	10 daily injections
0·5	7/7		
1·0	7/7	7/7	
2·5	7/7	7/7	
5·0	4/7	7/7	5/5
7·5	0/7	7/7	
10·0			0/10
15·0		0/7	
20·0			0/5
approximate LD50	5 mg/kg	10 mg/kg	7·5 mg/kg

Influence of glucose on tumour response

Values of the mean radiation dose in rad required to give local control of the tumour for 130 days (i.e. TCD–50/130), with 95% confidence limits, were 6800 (6370–7260) for animals which drank 10% glucose and 6240 (6060–6430) for animals which drank water. The difference between these values appears to be significant ($p < 0{\cdot}02$). Such a result is surprising in view of the opposite effect reported by other investigators [14]; however, the above values of TCD–50 lie at opposite ends of a range that has been found for untreated tumours in other experiments, and it should not be concluded from one experiment that administration of glucose can reduce the effectiveness of tumour irradiation. Certainly, however, there is no potentiation of the effect of irradiation of the present tumour by glucose administration.

Influence of TEM on tumour response

For mice injected with TEM and given a single dose of radiation, values of TCD–50/130 in rad (and their 95% confidence limits) were 6340 (5790–6950) for mice which drank 10% glucose, and 6160 (5440–6980) for mice which drank water. These values are not significantly different from the value of 6240 rad obtained for control mice. When irradiation was given as 10 daily fractions, values of TCD–50/130 were 11,260 (10,250–12,380) rads for mice injected with TEM and 11,700 (11,220–12,190) rads for mice injected with saline; again, these values are not significantly different. Thus, injection of TEM did not influence the probability of obtaining permanent local control of the present tumour by irradiation.

In Table 2 we have listed values of TCD–50/x for both single and fractionated doses of radiation, and for x = 60, 80, 100 and 130 days. Doses of radiation required to achieve local tumour control for periods of less than 130 days are significantly less in animals injected with TEM. The drug therefore causes a delay in the mean time to tumour regrowth. The mean duration of this delay at different levels of cell survival may be estimated from the horizontal displacement between the curves of Fig. 1; here, values of TCD–50/x are plotted against x

Table 2. *Values of TCD–50/x computed for local control of tumours for various times (x days) after irradiation*

x (days)	TCD-50/ x (95% confidence limits) in rad		Significance of difference
	TEM	Saline	
A: Single dose (pooled data for animals drinking water or glucose)			
60	4680 (4250–5150)	5500 (5200–5820)	$p < 0{\cdot}001$
80	5220 (4740–5760)	6120 (5670–6600)	$p < 0{\cdot}005$
100	5480 (5000–6010)	6320 (6080–6570)	$p < 0{\cdot}005$
130	6240 (5840–6670)	6560 (6330–6790)	not significant
B: 10 equal daily fraction			
60	8970 (8500–9460)	10,460 (9880–11,060)	$p < 0{\cdot}0005$
80	9620 (9030–10,240)	11,360 (10,900–11,850)	$p < 0{\cdot}0005$
100	10,300 (9630–11,010)	11,590 (11,070–12,140)	$p < 0{\cdot}005$
130	11,260 (10,250–12,380)	11,700 (11,220–12,190)	not significant

for animals injected with TEM or saline. After high single doses of radiation the mean delay to recurrence is about 35 days; after radiation given as 10 fractions it is about 50 days.

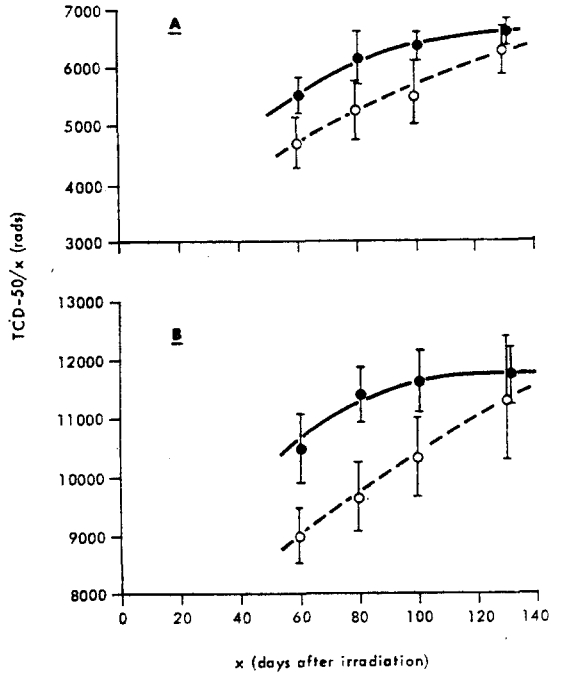

Fig. 1. TCD-50/x is plotted against x for (A) single dose irradiation and (B) irradiation given as 10 equal fractions. Mean values and 95% confidence limits are indicated for mice injected with TEM (open circles and dashed line) or saline (closed circles and solid line).

Influence of TEM on skin response

Mean doses of radiation to give skin responses in 50% of mice are listed in Table 3. There are no significant differences in the four pairs of values for TEM-injected and control mice.

Table 3. Influence of injection of TEM on skin response to irradiation

Response	Mean dose in rads (with 95% conf. limits) to give response in 50% of mice	
	TEM	Saline
3+Desquamation at 23 days after single dose	4330 (4000–4700)	4150 (3720–4630)
4+Epilation at 30 days after single dose	4590 (3960–5310)	4330 (3730–5010)
3+Desquamation at 29 days after 1st of 10 doses	8500 (7540–9570)	8790 (7800–9910)
3+Epilation at 31 days after 1st of 10 doses	6310 (4970–8020)	5860 (3300–10,400)

Values were computed for the times after irradiation when the reactions were most severe, and these times were also not influenced by TEM.

Spleen colony assays

Cell survival curves for the action of TEM on non-proliferating CFU in normal mice, and on proliferating CFU in chimeras are shown in Fig. 2. Proliferating CFU are more sensitive to the drug. Both sets of data may be represented by straight lines on a semilog plot, and values of D_0 (i.e. the dose required to reduce survival by 37%) are 0·58 mg/kg for non-proliferating CFU and 0·25 mg/kg for proliferating CFU (ratio of D_0-values = 2·3).

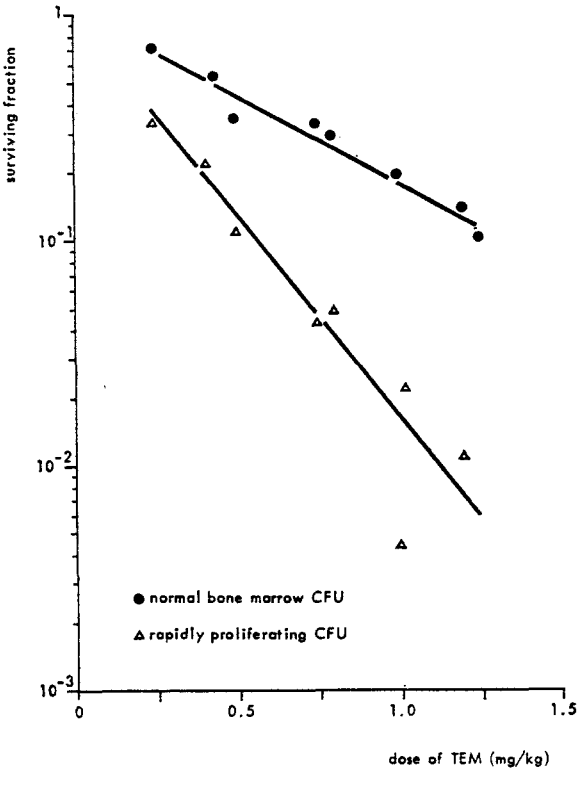

Fig. 2. Survival curves for the action of TEM on non-proliferating CFU in normal bone marrow and on proliferating CFU in the spleens of chimeras.

DISCUSSION

Our results show that supportive chemotherapy with TEM caused delay in regrowth, but no significant reduction in the dose of radiation required to obtain permanent local control of a C3H mouse mammary tumour. Thus there appears to be little therapeutic advantage in using TEM and radiation in the treatment of this tumour.

In other studies "percent increase in survival time" has often been used as an index of the effectiveness of an anti-cancer drug. Although

this parameter has been most useful and dependable in many studies, the present results demonstrate that it can be misleading: we obtained with chemotherapy a significant delay in time to tumour recurrence after irradiation (and an increase in mean survival time) but no increase in the probability of permanent tumour control.

High doses of radiation were used in the present study, and any recurring tumours must have originated from surviving cells that were severely hypoxic at the time(s) of irradiation. Thus our endpoint was a direct assay of the effect of TEM on these hypoxic cells. Our spleen colony assays demonstrate that non-proliferating bone marrow CFU are 2 to 3 times as resistant to TEM as proliferating CFU. Hypoxic cells in the present tumour are predominantly non-cycling [4] and therefore are probably also relatively resistant to TEM. However, if hypoxic tumour cells had even the same sensitivity to TEM as non-proliferating CFU, and if the drug concentration in their environment were the same, then the doses of TEM used should have reduced the survival of hypoxic tumour cells to less than 1%; the corresponding saving in radiation dose (without any selective activation of TEM due to low pH) should have been at least 1200 rad. Since we found no such dose reduction, the tumour cells must be intrinsically resistant to TEM, or the concentration achieved in the environment of hypoxic cells must be low.

The long delay in time to tumour recurrence observed in animals injected with TEM indicates that the drug leads to non-lethal effects on hypoxic cells. The observed delay might be due to an increase in the time between treatment and recruitment of originally non-proliferating hypoxic cells into cycle, or to a high rate of cell death among the progeny of such cells (i.e. clones derived from surviving cells might be smaller than in untreated tumours).

A significant potentiation in the response of other transplanted tumours to irradiation has been observed when glucose was infused intravenously and mice were given supplementary injections of insulin [14]. We did not use these methods because our assay involved large numbers of animals that were already being stressed by multiple injections. Our failure to demonstrate potentiation by glucose of response to irradiation might have been due to insufficient blood levels of glucose, or to inefficient passage of glucose into tumour cells; however, mice which drank 10% glucose gained body weight compared to controls.

Our results illustrate the difficulty of sterilizing hypoxic tumour cells, either with radiation or with drugs. Although our studies with TEM are largely disappointing, we believe that the approach outlined in the present paper is worthy of further study. Several other drugs are selectively activated or selectively deposited in regions of low pH [7–10]. We suggest that the sensitivity of tumour cells to drugs of this type should first be assayed, and that the more potent agents should be assessed for use with radiation using the type of endpoint that we have described. An improved method of attacking the hypoxic tumour cell seems to be fundamental in any approach to improving radiation therapy. Any specific property of this cell must be fully exploited.

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The Effect of Certain Nitrogen Mustard Derivatives on Bone Marrow Colony-Forming Units and Erythroid Repopulating Ability

I. Rats

C. D. R. DUNN*

Post Doctoral Fellow, Division of Biophysics, Institute of Cancer Research, Clifton Avenue, Belmont, Sutton, Surrey, England

Abstract—*The effect of two aliphatic (HN2 and HN3) and three aromatic (aminochlorambucil, melphalan and merophan) nitrogen mustard derivatives on rat bone marrow stem cells in different proliferative states has been investigated using both the spleen colony and erythroid repopulating ability assays. The results show that the effects of HN2 and HN3 are heavily dependent on the stem cell proliferation rate although this is relatively unimportant in determining the stem cell depression produced by melphalan and merophan. The reasons for these differences are discussed. It is concluded that differences in tissue distribution and/or access to target sites within the stem cells are responsible.*

The stem cell depression produced by aminochlorambucil is heavily dependent on the cell proliferation rate if the CFU assay is used, but changes in the cell proliferation rate do not influence the depression in the erythroid repopulating ability assay. These results can be interpreted as showing that the action of this agent is intermediate between the aliphatic and other aromatic derivatives, and are consistent with the suggestions that the cell population measured by the erythroid repopulating ability assay is different from the CFU.

A SURVEY of the published literature regarding the effects of certain nitrogen mustard derivatives on "stem cells"† yielded results which were apparently contradictory. Thus Bruce *et al.* [2] using the spleen colony assay technique in mice (with bone marrow as a source of normal and a different cell line, i.e. a lymphoma, as a source of rapidly proliferating stem cells) suggested that the effect of the aliphatic nitrogen mustard, HN2, was independent of cell proliferation rate. However, Blackett [3] using the erythroid repopulating ability technique with marrow taken from continuously irradiated mice as a source of rapidly proliferating stem cells has shown a small difference in the effect of a single dose of HN2 which suggests that the cell proliferation rate may play a part in the response of stem cells to this agent. Similar findings have been recently reported by other authors [4] for HN2, and the aromatic mustard derivatives,

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*Present Address: The Department of Haematology, The Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XN, Wales.

†In the context of this paper the term "stem cells" is used for convenience to denote those population(s) of cells in the bone marrow, proliferation of which is assayed by transplantation experiments, e.g. the spleen colony and erythroid repopulating assays. It does not infer that either assay necessarily measures cells with the properties usually ascribed to stem cells (e.g. self maintenance) except possibly their proliferative capacity. Nor is it inferred that the population measured by the spleen colony assay (the Colony-Forming Units, CFU) is necessarily the same as or different from that population measured by the Erythroid Repopulating Ability (ERA) [1].

chlorambucil and melphalan, using the spleen colony technique and exponentially doubling CFU in the spleen or bone marrow as a source of rapidly proliferating stem cells.

In rats, Harriss and Aponte [5] using the erythroid repopulating ability as a measure of stem cells and changes in the bone marrow cellularity as an indication of effects on rapidly proliferating cells, suggested that the effect of the aliphatic mustard, HN3, was dependent on cell proliferation rate. In addition Dunn and Elson [6] have shown that the aromatic nitrogen mustard derivative, aminochlorambucil, despite a large effect on bone marrow cellularity, had little or no effect on CFU. However, the closely related chemical, merophan, markedly depressed CFU in doses equitoxic to those of aminochlorambucil [7, 8]. Further, it has been shown [9] that bone marrow taken from chlorambucil-treated donors is more effective in keeping lethally irradiated rats alive than marrow from untreated animals. This finding could be interpreted as being due to a stem cell "sparing" effect of this chemical in rats concentrating the number of stem cells in a given bone marrow cell inoculum.

As the nitrogen mustard derivatives have an important place in cancer chemotherapy, the experiments reported in this and the following paper were designed to investigate more fully their mode of action. In particular, three points were of interest:

(1) Whether two aliphatic (HN2 and HN3) and three aromatic (aminochlorambucil, melphalan and merophan) nitrogen mustard derivatives, all with the same functional group, behaved similarly under a given set of conditions.

(2) Whether there was a difference between the spleen colony and erythroid repopulating ability techniques as measures of the effects of these mustards on stem cells.

(3) Whether there was a difference between rats and mice with respect to the action of these agents.

MATERIAL

(1) *Chemicals*

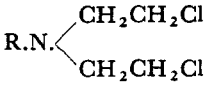
The chemical formulae of the five nitrogen mustard derivatives under investigation are shown in Table 1.

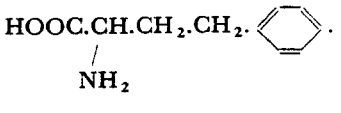
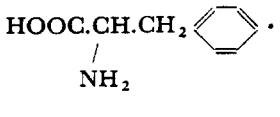
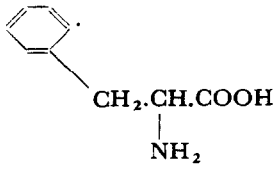
HN2 was obtained from Boots Pure Drug Co. Ltd., Nottingham, England and HN3 from the Schuchardt Chemical Co., Munich, Germany.

Busulphan, B.P., (1:4 dimethanesulphonoxybutane) was obtained from Burroughs Wellcome Ltd., and tritiated thymidine (^{3}H-TdR), 22 Ci/mM from the Radiochemical Centre, Amersham.

All other chemicals were supplied by the Chemistry Department of the Chester Beatty Research Institute.

Table 1. *Structural formulae of the nitrogen mustard derivatives under investigation.*



R =	Approved name	Chemical name
CH_3.	HN2	Di-2-chloroethylmethylamine hydrochloride
$Cl.CH_2.CH_2$.	HN3	Tri-2-chloroethylamine hydrochloride
	Aminochlorambucil	α-amino-[*p*-(di-2-chloroethylamino)-phenyl] butyric acid
	Melphalan	L-*p*-(Di-2-chloroethylamino) phenylalanine
	Merophan	D-*o*-(Di-2-chloroethylamino) phenylalanine

(2) *Animals*

Female, pure line Wilkie Hooded Rats, aged 6 to 8 weeks at the start of experimentation, were used throughout.

METHODS

(1) *Chemicals*

Busulphan and dimethyl myleran (dMM, 1:4 dimethyl dimethanesulphonoxybutane) were dissolved as previously described [10].

HN2 and HN3 were dissolved in saline.

Aminochlorambucil and melphalan were dissolved in absolute ethanol containing 2% concentrated hydrochloric acid and merophan in absolute methanol. These solutions were diluted 10 times with saline before injection.

For the study of the effect of the nitrogen mustard derivatives on stem cells, these chemicals were administered, intravenously, to the donor rats 24 hr before the marrow was taken for assay. Control animals received the solvent only.

All drug solutions were prepared immediately before use and the drug concentrations were such that a constant volume of 1 ml/100 g body weight of rat was injected.

(2) *Animals*

At least five recipients and between three and ten donor rats were used per experimental group.

(a) Spleen colonies: the method for the production of spleen colonies in chemically prepared rats has been fully described previously [6].

(b) Erythroid repopulating ability: (ERA) the assay for bone marrow stem cells using ^{59}Fe in rats prepared with dMM was that described by Blackett [11] with the exception that ^{59}Fe was injected on day 8 after bone marrow transplantation rather than day 7. The later time was preferred as a more adequate control ^{59}Fe uptake was obtained in the strain of rat used in the present investigation, and it also allowed a direct comparison between the results obtained with this technique and the 8-day spleen colony assay.

(c) Rapidly proliferating stem cells were obtained as described previously [6, 10] by treating donor rats 13 days before the mustard derivative with 10 mg/kg busulphan by intraperitioneal injection. Control rats for these experiments received busulphan 13 days before the solvent used for the mustard under investigation.

It has been previously shown using both the thymidine suicide technique [10] and vinblastine [6, 10] that the bone marrow CFU are proliferating more rapidly 13 days after busulphan than in control marrow. The results reported below also show that under these conditions the cell population measured by the ERA assay was proliferating more rapidly than normal.

(d) *In vitro* thymidine suicide was determined by the usual technique [10, 12].

(e) Experimental plan: At least four doses of each mustard derivative were used to determine each dose/response graph. In order of increasing dose let these be represented as A, B, C and D. "A" produced approximately 20 to 30% depression of the bone marrow cellularity and D was approximately equal to twice the LD_{50} based on death due to intestinal damage. B and C were intermediate doses.

At least three experiments were carried out to obtain each dose/response relationship. The first experiment investigated the effect of doses A and C, the second B and D and the third any other combination of two doses. Further experiments repeating some or all doses were carried out where necessary.

(f) Calculation and expression of results: For the both the CFU and ERA assays the results are expressed relative to one femur. The means and standard errors were calculated from the individual results in each group of recipients. The means and standard errors of the experimental groups are expressed as fractions of the relevant control value $\pm$ its standard error. Thus the points on the following figures represent the surviving fractions of control $\pm$ an error which includes the variation in the control group. These errors, therefore, represent the internal variation within the recipient groups (not the donors) and are a measure of the accuracy of the assay.

All lines on the figures have been drawn by eye.

RESULTS

Figures 1 to 5 show the effect of HN2 (Fig. 1), HN3 (Fig. 2), aminochlorambucil (Fig. 3), melphalan (Fig. 4) and merophan (Fig. 5) on both slowly and rapidly proliferating stem cells. The left hand side of the figures show the effects as measured by the spleen colony technique and the right hand as measured by the ERA assay.

Figures 1 and 2 show that HN2 and HN3 produce a greater depression of rapidly proliferating stem cells than slowly proliferating cells, i.e. the effect of these agents is markedly dependent on the rate of stem cell proliferation. The responses in both the CFU and ERA assays are essentially the same although HN3 may produce a slightly greater depression of normal bone marrow ERA than of normal bone marrow CFU.

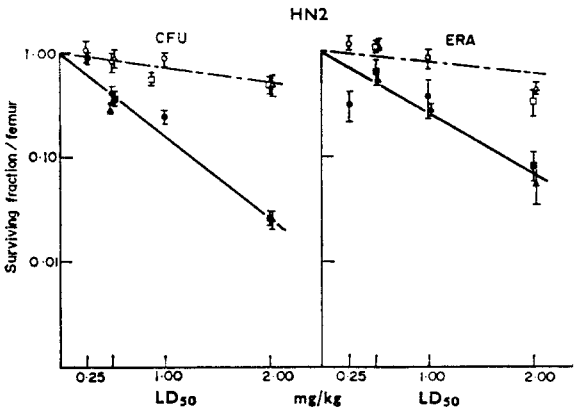


Fig. 1. The effect of HN2 on rat bone marrow stem cells. The left hand side shows the effects on CFU, the right on ERA.

Dashed line and open symbols = normal bone marrow stem cells.

Solid line and closed symbols = rapidly proliferating bone marrow stem cells.

Different shaped symbols represent the results of replicate experiments.

Vertical bars = ± one standard error of the mean.

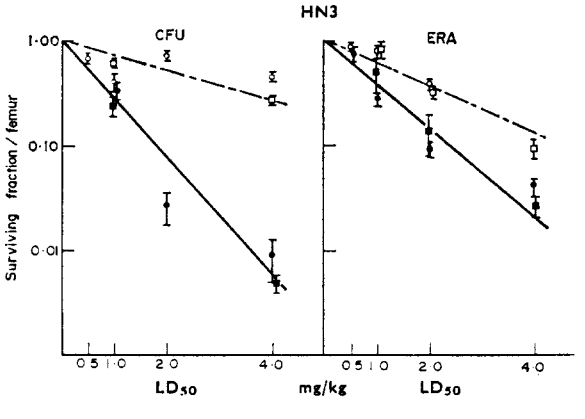


Fig. 2. The effect of HN3 on rat bone marrow stem cells. The left hand side shows the effects on CFU, the right on ERA.

Dashed line and open symbols = normal bone marrow stem cells.

Solid line and closed symbols = rapidly proliferating bone marrow stem cells.

Different shaped symbols represent the results of replicate experiments.

Vertical bars = ± one standard error of the mean.

For the aromatic mustard, aminochlorambucil (Fig. 3) there is a much greater effect on rapidly proliferating CFU than on normal bone marrow CFU. However, the depression of bone marrow ERA by aminochlorambucil is independent of the proliferation rate of this cell population.

Both the CFU and ERA assays suggest that the effect of the other aromatic mustards under investigation, melphalan (Fig. 4) and merophan (Fig. 5), is dependent to only a very small extent on the proliferation rate of the stem cells.

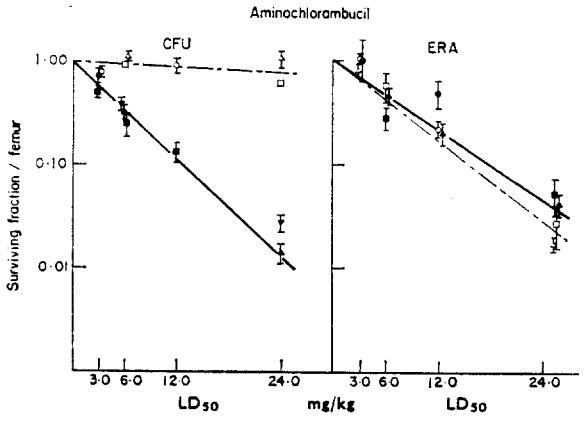


Fig. 3. The effect of aminochlorambucil on rat bone marrow stem cells. The left hand side shows the effects on CFU, the right on ERA.

Dashed line and open symbols = normal marrow stem cells.

Solid line and closed symbols = rapidly proliferating bone marrow stem cells.

Different shaped symbols represent the results of replicate experiments.

Vertical bars = ± one standard error of the mean.

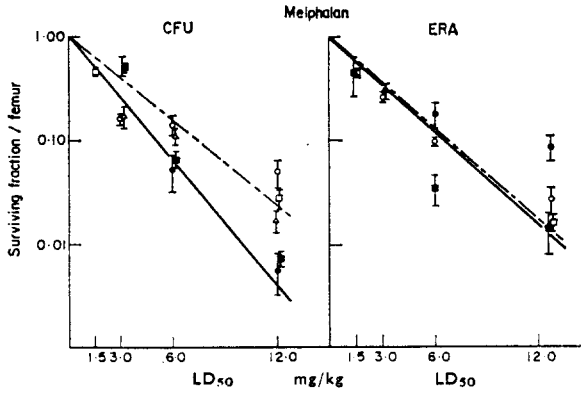


Fig. 4. The effect of melphalan on rat bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA.

Dashed line and open symbols = normal bone marrow stem cells.

Solid line and closed symbols = rapidly proliferating bone marrow stem cells.

Different shaped symbols represent the results of replicate experiments.

Vertical bars = ± one standard error of the mean.

Table 2 shows the effect of 45-min incubation with tritiated thymidine on normal bone marrow stem cells and on stem cells 13 days after busulphan. Within the experimental

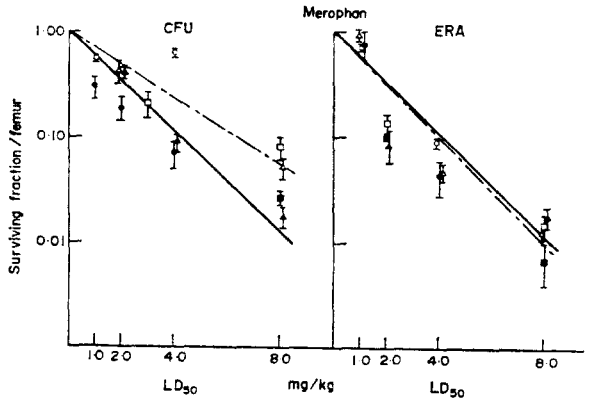


Fig. 5. The effect of merophan on rat bone marrow stem cells. The left hand side shows the effects on CFU, the right on ERA.

Dashed line and open symbols = normal bone marrow stem cells.

Solid line and closed symbols = rapidly proliferating bone marrow stem cells.

Different shaped symbols represent the results of replicate experiments.

Vertical bars = ± one standard error of the mean.

limits there is no difference in the depression of normal bone marrow CFU or ERA and these results provide further confirmation that the CFU 13 days after busulphan are proliferating more rapidly than normal. However, they suggest that 13 days after busulphan the proliferation rate of the cells measured by the ERA assay is not as rapid as the CFU although a similar degree of stem cell recovery (to 30–40% of normal) after busulphan is observed with both assays. This apparent slower proliferation rate of the cell population measured by the ERA assay compared to the CFU 13 days after busulphan may explain why the mustards produce rather less depression of the ERA assay than of the CFU recovering from busulphan treatment.

DISCUSSION

The results presented show that the proliferation rate of the stem cells is important to varying degrees in determining the effect of the nitrogen mustard derivatives. Thus, with the aliphatic mustards, HN2 and HN3, changes in proliferation rate markedly alter the response of the stem cells to these agents. Changes in proliferation rate are less important, if at all, in the response of bone marrow stem cells to the aromatic mustards melphalan and merophan. For equitoxic doses, as assessed by intestinal damage, this difference is due to the relatively greater effect on normal bone marrow stem cells of melphalan and merophan compared to that of the aliphatic mustards, HN2 and HN3. These results are in general agreement with those obtained in less extensive investigations [5, 6, 8].

The aromatic nitrogen mustard derivative aminochlorambucil appears to be intermediate in action between the aliphatic and the other aromatic agents and will be discussed below.

The most likely reasons for the difference in effects between HN2 and HN3 on the one hand and melphalan and merophan on the other are:

(a) a difference in tissue distribution;

(b) a difference in access to target sites in the stem cells;

(c) a difference in the chemical reaction mechanism which may influence access of the agents to the target sites.

(a) *Differences in tissue distribution*

One of the limiting factors in the clinical or experimental use of these agents is intestinal toxicity and it is this criterion which has been employed to select the doses used in the present investigation. If there were a difference in the

Table 2. The surviving fractions (± one standard error of the mean) of stem cells per femur of normal bone marrow or bone marrow thirteen days after busulphan, after 45 min incubation with tritiated thymidine. The surviving fractions are calculated with reference to the control samples of bone marrow which were incubated with equivalent concentrations of "cold" thymidine.

Mean of two experiments in which both CFU and ERA assays were carried out on the same bone marrow sample.

Concentration of tritiated thymidine	Surviving fractions			
	CFU/femur		ERA/femur	
	Normal bone marrow	13 days after busulphan	Normal bone marrow	13 days after busulphan
250 μCi/ml	1·02 ± 0·07	0·42 ± 0·07	0·90 ± 0·23	0·71 ± 0·17
500 μCi/ml	0·91 ± 0·09	0·21 ± 0·03	1·29 ± 0·25	0·50 ± 0·12

relative distribution between the intestine and the bone marrow for the aliphatic and aromatic mustards then it might be expected that, for a dose of given intestinal toxicity, (e.g. LD_{50}) the bone marrow effects would be different. A difference in the relative distribution of HN2 and melphalan has been demonstrated using radioactively labelled chemicals. Six to 24 hr after injection of HN2 approximately 20 times more radioactivity is found in the intestine than in the bone marrow [13], while after melphalan the difference in distribution of radioactivity is only 2 to 3 fold [14]. Thus, if it is assumed that the radioactivity measures the active drug concentration in the tissues, then, for doses equitoxic in terms of intestinal damage, it might be expected that melphalan would have a greater effect on the bone marrow than HN2, in agreement with the results shown in Figs. 1 and 4.

While changes in the relative distribution of the aliphatic and aromatic nitrogen mustards may possibly explain the differing effects on normal bone marrow stem cells, it is unlikely that distribution differences can account for the varying degrees of dependency on target cell proliferation rate. Furthermore, it has been observed (see also the following paper) that for a given depression in the bone marrow cellularity, which could be used as an indication of the effective concentration of these agents in the bone marrow, there are large differences in the effects on stem cells between the aliphatic and aromatic derivatives. These findings suggest that differences in the relative distribution may not be as important as:

(b) *a difference in access to target sites in the stem cells*

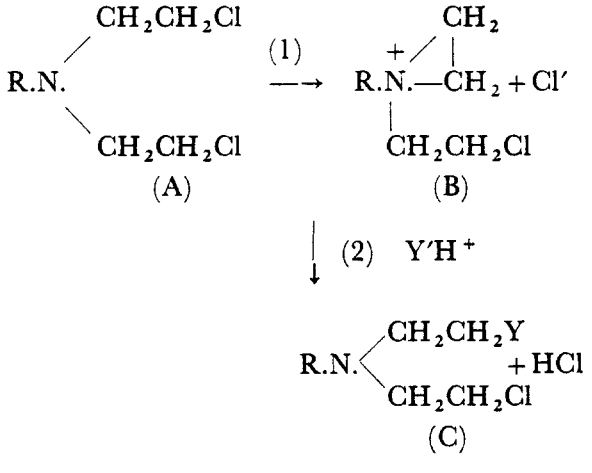
It has recently been shown *in vitro* that the aliphatic mustard HN2 is actively taken up into L5178Y cells by the choline transport mechanism whereas the aromatic derivatives melphalan and chlorambucil enter by diffusion only [15]. If the rate of active transport varies with the cell proliferation rate but the rate of diffusion is not affected then the difference, now reported, in the degree to which the aliphatic and aromatic mustards are proliferation dependent could be explained. As this hypothesis assumes that normal bone marrow stem cells are freely permeable to the aromatic mustards, then the data presented in Table 3, which show that the aromatic mustards are more potent than the aliphatic derivatives in producing depression of normal bone marrow stem cells, are consistent with this theory.

A major factor in the penetration of chemicals through cell membranes is the ionic state of the molecules. As a general rule ionized compounds enter cells by some type of active transport system while the lipid soluble, non-ionized forms enter by diffusion. Thus a difference in the ionic state of the aliphatic or aromatic mustards could determine both the mechanism and rate of entry of these agents into the cell.

Table 3. The doses (μM) of the nitrogen mustard derivatives required to reduce survival of normal rat bone marrow stem cells to 50% of control levels ($D_{0.5}$).
* *The values are calculated fron Figs. 1–5.*

	$D_{0.5}$	
	CFU/femur	ERA/femur
HN2	13·0	13·0
HN3	11·0	8.6
Aminochlorambucil	>72	18·6
Melphalan	6·6	6·6
Merophan	6·6	6·6

(c) *the chemical reaction mechanism*



where Y′ is a nucleophilic reagent, has been shown to be common to both aliphatic and aromatic mustards [16]. For HN2, step (1) which takes place rapidly in aqueous media, is faster than step (2) while the reverse is the case for aromatic derivatives such as melphalan. Thus, these findings are in agreement with the observations [15], discussed above, that active transport of the ionized form of HN2 (B) is necessary for the passage of this agent across the cell membrane, while diffusion of the lipid soluble, non-ionized, parent compound (A) is sufficient for penetration of melphalan through the cell membrane.

At the present time a difference in the tissue distribution and/or slight variations in the ionic state of the molecule which profoundly in-

fluences cell penetration seem the most likely explanations for the different effects of the aliphatic and aromatic nitrogen mustard derivatives. The effects of aminochlorambucil are more difficult to explain.

Although aminochlorambucil is an aromatic mustard derivative, its effects on bone marrow stem cells can be considered as intermediate between the aliphatic derivatives HN2 and HN3 and the other aromatic chemicals melphalan and merophan. With the CFU assay, the effect of aminochlorambucil is found to be heavily dependent on the cell proliferation rate (Fig. 3) and, thus aminochlorambucil resembles the aliphatic derivatives more closely than the aromatic. Cell proliferation rate is less important in the response of ERA to this agent (Fig. 3) and thus aminochlorambucil behaves similarly to the other aromatic derivatives. The difference between the results obtained with these two assays will be discussed more fully below.

Although chemically aminochlorambucil, and the closely related agent, chlorambucil, are aromatic mustards, the possibility that they can behave as aliphatic derivatives is suggested by the following:

(i) In the chemical reaction outlined above, chlorambucil is similar to the aliphatic mustards and unlike the other aromatic derivatives, since Step (1) is faster than Step (2) [ref. 16]. The importance of this reaction with regard to cell penetration has yet to be determined but ionization of the mustard derivative with the formation of the ethyleneimmonium ion (B) would be expected to reduce lipid solubility and, hence, diffusion of chlorambucil across cell membranes. As active transport of this agent, at least by the choline transport mechanism, is unlikely [15], ionization may account for the fact that aminochlorambucil is less potent in producing depression of normal bone marrow CFU than the other mustard derivatives investigated (Table 3).

(ii) Bone marrow from chlorambucil-treated donors is more effective than marrow from untreated donors in keeping alive lethally irradiated rats [9]. This would suggest a stem cell "sparing" effect of chlorambucil, i.e. similar to the aliphatic mustards and unlike the other aromatic derivatives melphalan and merophan.

(iii) It has also been observed [6, 17] that, like HN2 and unlike melphalan and merophan [18], aminochlorambucil and chlorambucil depress peripheral blood lymphocytes to a greater extent than the neutrophils.

With the possible exception of HN3 only after aminochlorambucil (Fig. 3) is there an appreciable difference between the CFU and ERA assays. As the CFU data imply that the proliferation rate is very important with respect to the effect of aminochlorambucil, a possible interpretation of the results is that in normal rat bone marrow the cell population measured by the ERA assay is proliferating more rapidly than the CFU. Although there is some evidence from studies in mice which is consistent with this conclusion [1, 19] the difference in proliferation rate is not confirmed by the thymidine suicide technique (Table 2) but this only gives information on the ratio of the period of DNA synthesis to the cell cycle time. In any case, it is unlikely that differences in cell cycle times of ERA and CFU can explain the different effects of aminochlorambucil since HN2, the effect of which is markedly proliferation dependent (Fig. 1), has no greater effect on normal bone marrow ERA than CFU. A more likely explanation is a biochemical difference between ERA and CFU which prevents access of aminochlorambucil to its target site in one cell population producing the sevenfold difference in potency with respect to the effect of this agent on CFU and ERA (Table 3).

The foregoing discussion on the differences between the CFU and ERA assays after aminochlorambucil presupposes that the two assays measure different cell populations [1, 19]. An alternative viewpoint is that aminochlorambucil is having a specific effect on the differentiation of a pluripotential stem cell into the erythroid cell line. This seems unlikely, however, since it has been shown [20] that treatment of the donors with aminochlorambucil has no effect on the histology of rat spleen colonies.

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The Effect of Certain Nitrogen Mustard Derivatives on Bone Marrow Colony-Forming Units and Erythroid Repopulating Ability

II. Mice

C. D. R. DUNN*

Post Doctoral Fellow, Division of Biophysics, Institute of Cancer Research, Clifton Avenue, Belmont, Sutton, Surrey, England

Abstract—*The effects of the same five nitrogen mustard derivatives as were used in the preceding paper, have been investigated on bone marrow stem cells in mice. All these agents behave in an essentially similar manner with no evidence that the effects are dependent on cell proliferation rate.*

Possible reasons for the difference between the effects of the aliphatic nitrogen mustards in rats and mice are discussed but one arrived at no entirely satisfactory conclusions.

It is pointed out that differences between mustard derivatives in one species and differences in the way different species behave to a given mustard, make estimation of the effect of these agents in humans difficult.

THE STUDIES reported in this paper were carried out concurrently with those described in the previous communication, so that a direct comparison between the effects of five nitrogen mustard derivatives on bone marrow stem cells in rats and mice could be made.

MATERIALS

(1) *Chemicals*

The sources of the various chemicals were as described previously [1].

(2) *Animals*

Male and female CBA mice, aged 8 to 10 weeks at the start of experimentation, were used throughout. Male mice were always used as donor animals, recipients were males and/or females.

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*Present address: The Department of Haematology, The Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XN, Wales.

METHODS

(1) *Chemicals*

These were dissolved and administered intravenously to the donor mice 24 hr before stem cell assay as described previously [1].

(2) *Animals*

At least five recipients and between two and ten donor mice were used per experimental group.

For the spleen colony or erythroid repopulating ability (ERA) assays, all the recipient mice were exposed to 750 rad whole body *x*-irradiation (230 kV, 15 mA, dose rate 25–30 rad/min, 1 mm Cu + 1 mm Al filtration), approximately 2 hr before receiving the bone marrow. The dose of irradiation was sufficient to reduce the mean endogenous colony count to less than 0·1 colonies/spleen.

(a) Spleen colonies: The assay for haemopoietic colony-forming units (CFU) in the spleen of recipient mice was the standard procedure of Till and McCulloch [2]. Eight days after bone marrow transplantation, the recipient mice were killed, the spleens removed

and fixed in Bouin's Solution and all macroscopic colonies on the flat surface of the spleen counted.

Rapidly proliferating CFU were obtained by the transplantation of 5×10^6 bone marrow cells (approximately 1000 CFU) into irradiated recipients according to the usual technique [3–5]. Seven days after bone marrow transplantation the CFU in both the spleen and femoral bone marrow of these primary recipients showed an increased sensitivity, compared to normal CFU, to vinblastine [3 and unpublished observations]. At this time, the nitrogen mustard derivatives or, in the control group, the solvent only were administered and the CFU content of the primary recipients' spleen or femoral bone marrow assayed 24 hr later by transplantation into secondary recipients. The number of macroscopic spleen colonies was assessed 8 days after the secondary transplantation according to the procedure outlined above.

(b) Erythroid repopulating ability: The method was that described by Twentyman and Blackett [6], modified slightly as reported in an earlier communication [7].

The effect of the mustard nitrogen derivatives on rapidly proliferating bone marrow ERA was not investigated for two reasons: a large number of animals was required and, secondly, the effect of HN2 and several other cytotoxic agents on normal and rapidly proliferating ERA was carried out in this laboratory at a similar time as the present investigations. The results will be reported in the near future [8].

(c) *In vitro* thymidine suicide was carried out by the standard method [9].

(d) Experimental plan: With one exception there is little difference in the toxicities of the five nitrogen mustard derivatives under investigation in rats or mice, so the effect of the same doses per unit body weight as were used in the preceding paper were now investigated for their effects on stem cells in the mouse. The same doses of melphalan as were used for the investigations in rats were also used in the experiments in mice reported below although there is a difference of a factor of two in the toxicity between the two species. Therefore, the highest dose of this agent (12 mg/kg), although it is twice the LD_{50} in rats, is approximately the LD_{50} in mice. As CFU survival in mice after this dose was less than 0·1% (see Fig. 4) it was considered that investigations using higher doses of melphalan were unnecessary.

(e) The results are calculated and expressed as in the previous paper [1].

RESULTS

Figures 1 to 5 show the effect of HN2 (Fig. 1) HN3 (Fig. 2), aminochlorambucil (Fig. 3), melphalan (Fig. 4) and merophan (Fig. 5) on mouse bone marrow stem cells. The left hand side of the figures shows the effect on normal bone marrow CFU and rapidly proliferating bone marrow derived CFU in either the spleen or bone marrow of primary recipients. The right hand side shows the effect on normal bone marrow ERA.

The responses of normal bone marrow CFU and ERA to HN2 (Fig. 1) were similar, showing an exponential decline over the dose range studied. There was no evidence of a greater effect on rapidly proliferating CFU compared to normal CFU.

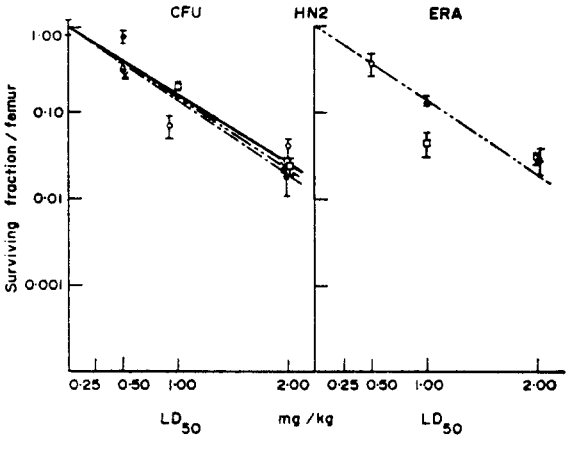

Fig. 1. The effects of HN2 on mouse bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA.

Open symbols and —— — —— = normal bone marrow stem cells. Closed symbols and solid lines = rapidly proliferating bone marrow derived CFU in the bone marrow.

Crosses and —— — —— = rapidly proliferating bone marrow derived CFU in the spleen.

Different shaped symbols represent the results of replicate experiments.

Vertical bars indicate ± one standard error of the mean.

With the doses used in these present studies, the other four nitrogen mustard derivatives (HN3, aminochlorambucil, melphalan and merophan) produced considerably greater depression of bone marrow stem cells than HN2. Furthermore, there are suggestions that stem cell depression after these derivatives may not decline exponentially with increasing dose although considerably more experimental points, at low stem cell survival levels, are necessary to confirm the shape of the lines drawn in Figs. 2 to 5. Thus, after HN3 (Fig. 2) the responses of normal bone marrow CFU and ERA and rapidly proliferating bone marrow derived CFU in the bone marrow of primary

recipients appeared to show a lack of exponential decline with increasing dose. This was not apparent with rapidly proliferating CFU in the spleen which decreased exponentially with increasing dose of HN3 to a CFU survival of less than 0·1%. There was no evidence of a greater effect on rapidly proliferating CFU than on normal CFU.

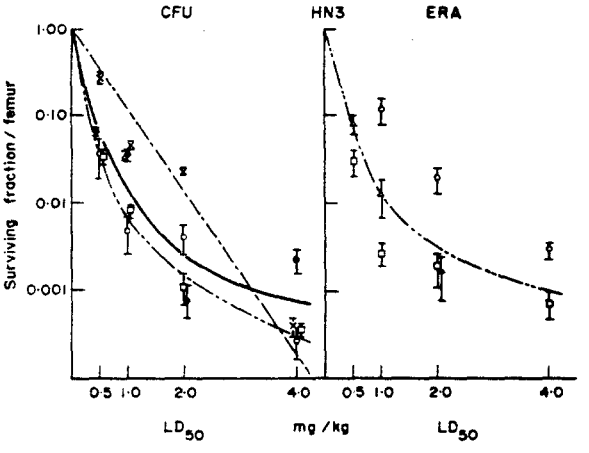

Fig. 2. The effect of HN3 on mouse bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA.

Open symbols and —— — — —— = normal bone marrow stem ells.

Closed symbols and solid lines = rapidly proliferating bone marrow derived CFU in the bone marrow. Crosses and —— — —— = rapidly proliferating bone marrow derived CFU in the spleen.

Different shaped symbols represent the results of replicate experiments.

Vertical bars indicate ± one standard error of the mean.

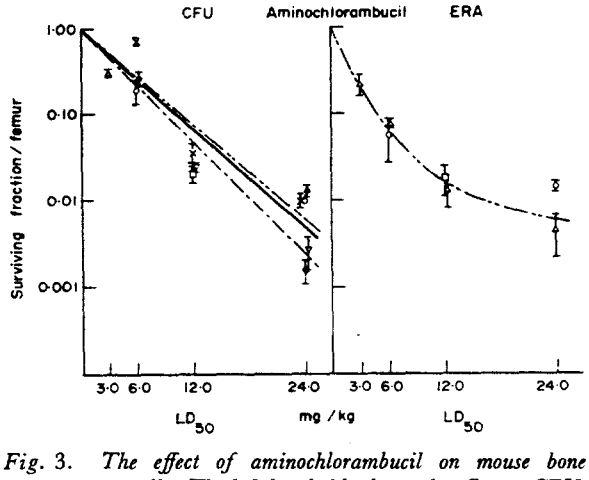

Fig. 3. The effect of aminochlorambucil on mouse bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA.

Open symbols and —— — — —— = normal bone marrow stem cells.

Closed symbols and solid lines = rapidly proliferating bone marrow derived CFU in the bone marrow. Crosses and —— — —— = rapidly proliferating bone marrow derived CFU in the spleen.

Different shaped symbols represent the results of replicate experiments.

Vertical bars indicate ± one standard error of the mean.

The response of all three CFU populations to aminochlorambucil (Fig. 3) showed exponential decreases with increasing dose with no evidence of differential effects on slowly and rapidly proliferating cells. There was evidence of a plateau in the ERA dose response graph at approximately 1% survival.

The effect of melphalan (Fig. 4) on CFU and ERA was essentially the same as that observed

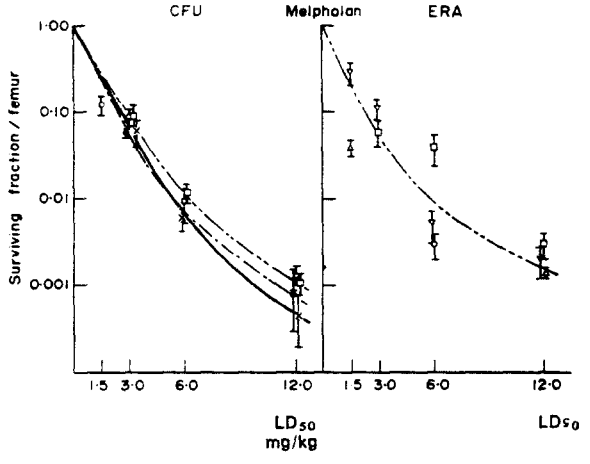

Fig. 4. The effect of melphalan on mouse bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA.

Open symbols and —— — — —— = normal bone marrow stem cells.

Closed symbols and solid lines = rapidly proliferating bone marrow derived CFU in the bone marrow. Crosses and —— — —— = rapidly proliferating bone marrow derived CFU in the spleen.

Different shaped symbols represent the results of replicate experiments.

Vertical bars indicate ± one standard error of the mean.

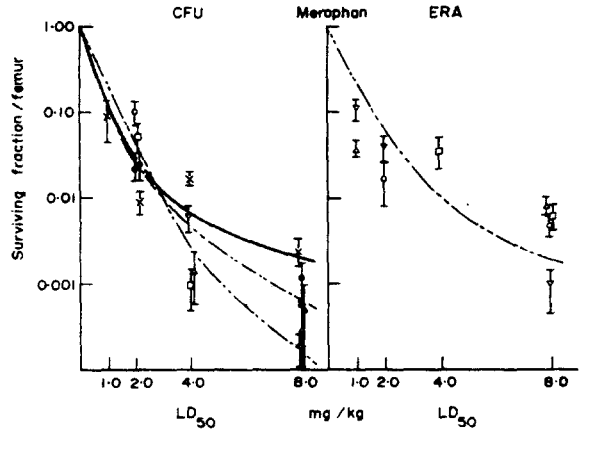

Fig. 5. The effect of merophan on mouse bone marrow stem cells. The left hand side shows the effect on CFU, the right on ERA. Open symbols and —— — — —— = normal bone marrow stem cells. Closed symbols and solid lines = rapidly proliferating bone marrow derived CFU in the bone marrow. Crosses and —— — —— = rapidly proliferating bone marrow derived CFU in the spleen.

Different shaped symbols represent the results of replicate experiments.

Vertical bars indicate ± one standard error of the mean.

following merophan (Fig. 5). None of the cell populations appeared to show an exponential decrease with increasing dose of these agents and there was little evidence of differential effects on slowly and rapidly proliferating CFU.

With the exception of aminochlorambucil, which is rather less potent than the other nitrogen mustard derivatives, both the aliphatic and other aromatic mustards are approximately equipotent (Table 1) and more potent than either group of derivatives in rats [1].

Similar to the situation in rats [1], within the experimental limits there was no difference in the response of normal mouse bone marrow CFU and ERA to 45 min incubation with high specific activity tritiated thymidine (Table 2).

A study of the above dose/survival curves of normal bone marrow CFU show that, similar to the situation in rats [1], for doses equitoxic in terms of intestinal death the CFU depression varied with the different mustard derivatives. Also, for a given nitrogen mustard derivative, equitoxic doses in rats and mice produced different depressions of mouse and rat bone marrow CFU and this may explain the apparent differences in the shape of the dose survival curves in the two species since the stem cells in rats were not reduced to the same low levels of survival as in mice. A

Table 1. The doses (μM) of the nitrogen mustard derivatives required to reduce survival of normal mouse bone marrow stem cells to 50% of control levels ($D_{0\cdot5}$). The values are calculated from Figs. 1 to 5

	$D_{0\cdot5}$	
	CFU/femur	ERA/femur
HN2	2·5	2·5
HN3	1·3	1·3
Aminochlorambucil	6·2	6·2
Melphalan	2·5	2·5
Merophan	2·5	2·5

Table 2. The surviving fractions (± one standard error of the mean) of normal mouse bone marrow stem cells after 45 min incubation with tritiated thymidine. The surviving fractions are calculated with reference to the control samples of bone marrow which were incubated with equivalent concentrations of "cold" thymidine.

Mean of two experiments in which both CFU and ERA assays were carried out on the same bone marrow sample

	Surviving fractions of Normal Bone Marrow	
	CFU/femur	ERA/femur
250 μCi/ml	0·87 ± 0·12	1·22 ± 0·25
500 μCi/ml	0·92 ± 0·09	1·10 ± 0·26

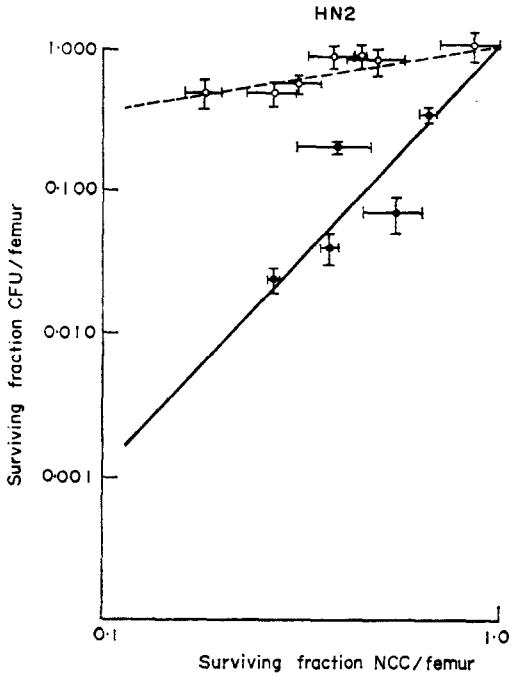

Fig. 6. The relationship between the surviving fraction of bone marrow nucleated cell count (NCC)/femur and CFU/femur after HN2 in rats (open symbols and broken line) and mice (closed symbols and solid line).

Bars indicate ± one standard error of the mean.

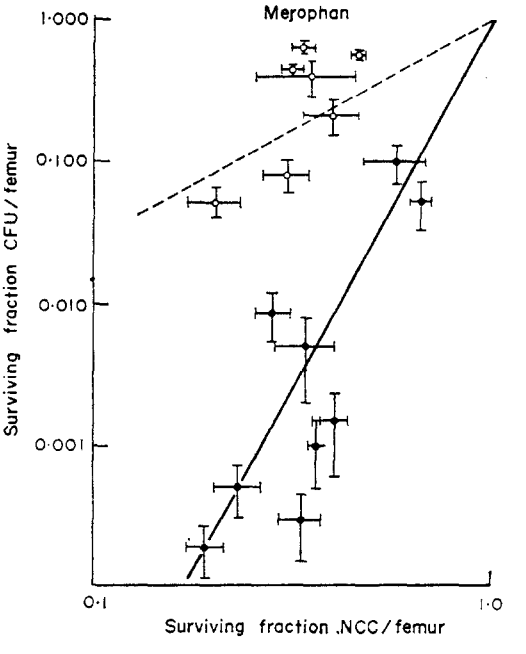

Fig. 7. The relationship between the surviving fraction of bone marrow nucleated cell count (NCC)/femur and CFU/femur after merophan in rats (open symbols and broken line) and mice (closed symbols and solid line).

Bars indicate ± one standard error of the mean.

direct comparison between the effects of two of the nitrogen mustard derivatives on normal rat and mouse bone marrow is shown in Figs. 6 and 7. The data for rats were obtained from the experiments reported in the preceding paper [1]. The surviving fractions of CFU/femur are plotted against the surviving fractions of bone marrow nucleated cell count (NCC)/femur for the various doses used in these investigations. In the context of these diagrams, therefore, the depression in the NCC/femur can be considered as showing that effective concentrations of these nitrogen mustard derivatives were obtained in the bone marrow of both rats and mice. For HN2 (Fig. 6) which, like HN3, had little effect on normal rat bone marrow CFU, for a given survival of bone marrow NCC, the CFU survival differed from rats to mice. For merophan (Fig. 7) which, like melphalan, produced considerable depression of normal rat bone marrow CFU, the difference in the CFU survival between rats and mice for a given survival of NCC is even more marked than for HN2.

DISCUSSION

The effects of all five nitrogen mustard derivatives on mouse bone marrow stem cells were essentially the same and, unlike the depression produced by the proliferation dependent cytotoxic agents cyclophosphamide and 5-fluorouracil [7], there was good agreement between the responses of normal bone marrow CFU and ERA.

In agreement with the results of Bruce *et al.* [10] there was little difference in the slopes of the graphs of normal or rapidly proliferating CFU to indicate proliferation dependence of the nitrogen mustards i.e. in mice all five derivatives appear to be Class I agents. However, the suggestion that several dose survival curves did not decrease exponentially with increasing dose of the nitrogen mustards may be interpreted as resulting from a resistant subpopulation of cells. This resistance could possibly be a consequence of a phase specificity of the nitrogen mustards which is the normal interpretation of the action of agents such as vinblastine [10] which produce a non-linear dose survival curve.

The demonstration that the effect of the five nitrogen mustard derivatives is independent of the cell proliferation rate is in contrast to the results obtained with HN2 and melphalan by other workers [5] using the same CFU assays and closely related strains of mice to those used in the present study. This difference is due to the fact that normal mouse bone marrow CFU of van Putten and Lelieveld [5] are not depressed to the same extent as in the investigations now reported. Van Putten and Lelieveld [5], however, used marrow taken from donor mice treated 16 hr previously with a subcutaneous injection of the mustards compared to 24 hr after an intravenous injection used in the present experiments. Preliminary investigations indicate that these different conditions can explain a large part of the discrepancies between the results now discussed and those of the Dutch workers.

The conclusion that the effect of HN2 in mice is independent of the cell proliferation rate also apparently differs from the conclusion reached by Blackett [8] who investigated the stem cell effects using the ERA technique under the same conditions of timing of the assay and route of administration of the mustard as were used in the present study. The bone marrow of mice which were continuously irradiated for two weeks was the source of rapidly proliferating stem cells which were slightly more sensitive to HN2 than normal bone marrow stem cells. The reason for this difference is not clear. Blackett's experiments were carried out in a different strain of mice to the present studies although there is good agreement in the dose survival curves of normal bone marrow ERA between the two strains. It is probable that the ERA assay does not measure CFU [7, 11] and the previous paper [1] shows that the degree to which at least one nitrogen mustard derivative, aminochlorambucil, is proliferation dependent depends on whether the ERA or CFU assay is used. Furthermore, there is considerable uncertainty [6, 12] regarding the degree to which stem cells in continuously irradiated mice are proliferating more rapidly than normal.

The results presented above show that the two aliphatic (HN2 and HN3) and the three aromatic (aminochlorambucil, melphalan and merophan) nitrogen mustard derivatives produce similar effects on mouse bone marrow stem cells. There is no evidence that these effects are dependent on target cell proliferation rate. These results contrast to the effects in rats [1] where the stem cell response to HN2 and HN3 is heavily dependent on cell proliferation rate and normal bone marrow stem cells are depressed by a small degree relative to intestinal toxicity or depression of bone marrow cellularity.

The different effect of HN2 on normal bone marrow in rats and mice is clearly shown in

Fig. 6. For a given survival of the bone marrow cellularity HN2 depresses CFU in mice to a lower level than CFU in rats. Even for merophan (Fig. 7) which markedly depresses normal rat bone marrow CFU, for a given survival of the bone marrow cellularity a differential effect on CFU between rats and mice is also observed. These results and the observations that the nitrogen mustards have different potencies in the two species, suggest a greater penetration of these agents into bone marrow stem cells in mice than in rats. Thus, the effect of the nitrogen mustards, particularly the aliphatic derivatives, in rats can be considered as "stem cell sparing". It seems unlikely that this effect is the result of variations in the effective level of the mustards in the bone marrow of the two species (Figs. 6 and 7). Also results with 5-fluorouracil [13] suggest that differences in availability times are unlikely to explain why HN2 and HN3 are proliferation dependent in rats but not in mice.

The results presented in this and the preceding paper [1] illustrate that five nitrogen mustard derivatives, all with the same alkylating moieties, do not behave in the same way in rats and mice with reference to the target cell proliferation rate. Apparent contradictions in the literature mentioned in the previous report [1] can be ascribed to these differences, which make extrapolation of the effects of nitrogen mustard derivatives between rodents and humans difficult. Furthermore the results indicate that differences between Class I and Class III agents may not be as definitive as was at first considered but may depend on species, assay system or even, possibly, the route and timing of administration.

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Effects of Methotrexate on Established Cell Lines of Human Choriocarcinoma*†

ROBERT O. HUSSA and ROLAND A. PATTILLO
Cancer Research and Reproductive Biology Laboratories, Department of Gynecology and Obstetrics, Medical College of Wisconsin, 8700 W. Wisconsin Avenue, Milwaukee, Wisconsin 53226

Abstract—*Cell lines of human choriocarcinoma (BeWo and Jar lines) were incubated in vitro for up to 7 days with 0·10, 10, or 1000 μM methotrexate (MTX). The cell population did not change significantly for up to 6 days after daily addition of 0·10 to 1000 μM MTX to the culture medium, that was also changed each day. During the same time period the cell population quadrupled in untreated control flasks. The drug caused an increase in amount of protein per cell and in the protein: DNA ratio. Incubation for 5 hr with MTX at concentrations of 0·10 and 10 μM resulted in 83 and 93% inhibition, respectively, of deoxyuridine-6-^3H incorporation into DNA. After 3 days of incubation with either 0·10 or 10 μM MTX, no incorporation of labeled deoxyuridine into DNA was observed. Incubation in the presence of the antifolate resulted in a rapid dose-dependent compensatory increase in incorporation of thymidine-2-^{14}C into DNA, rising to 135 and 197% of untreated control levels after 24 hr of incubation with 0·10 and 10 μM MTX, respectively. At later times a decrease occurred in absolute rate of thymidine incorporation into DNA, but not in the relative rate compared to untreated cells of the same age. MTX did not significantly alter the incorporation of leucine-^3H or uridine-^3H into the trichloroacetic acid precipitate, nor of glucose-^3H into DNA, RNA, protein, or lipid in the trophoblastic cells. Omission of serum from the daily medium did not change the above results. Unsuccessful attempts were made to subculture cells previously incubated with concentrations of 0·10 μM MTX or greater, although successful subculturing was possible in the presence of 0·01 μM MTX. The deleterious effects of MTX observed in both the BeWo and Jar cell lines were consistent with the suggestion that the major effect of the drug is to inhibit dihydrofolate reductase.*

INTRODUCTION

PRIOR to the advent of chemotherapy, invasive trophoblastic tumors were almost always fatal. In 1964 Hertz *et al.* [1] reported that 52% of patients treated with MTX for metastatic trophoblastic disease obtained complete remission without relapse for 1 to 7 years. However, it is well documented that trophoblastic tumors frequently become resistant to MTX [2, 3]. From the foregoing we considered it important to investigate the detailed mechanism of action of MTX on malignant trophoblastic cells.

The availability of a trophoblastic cell line (BeWo) in which the characteristics of the original tumor (malignancy, hormone production, aneuploidy) have been maintained for nearly 6 years [4, 5] provides an ideal model system with which to study the effects of chemotherapeutic agents [6–8]. This cell line came from the MTX-resistant choriocarcinoma of a patient who succumbed to her disease. The tumor was transplanted and maintained serially in the hamster by Hertz [9]. In 1966

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The abbreviations used are: BSS, Gey's balanced salt solution; MTX, methotrexate; TCA, trichloroacetic acid.

the tumor was established in tissue culture by Pattillo and Gey [4]. Preliminary studies in this laboratory [7] documented the dose-dependent inhibition of tritium-labeled deoxyuridine incorporation into DNA after treatment of BeWo cells with MTX. The present investigation examines in greater detail these actions of MTX, as well as the sensitivity of other gross biosynthetic pathways to the drug in trophoblastic BeWo cells. In addition, similar studies have been performed on another trophoblastic cell line (Jar), also obtained from a patient whose choriocarcinoma had become resistant to MTX [8, 10]. There are numerous reports of the deleterious effect of MTX on benign and malignant trophoblast *in vivo* [1–3, 11, 12]. However, except for a brief preliminary study [7], this is the first report on the effects of MTX on the malignant human trophoblast in continuous cell culture.

MATERIAL AND METHODS

The human trophoblastic cell lines studied were BeWo [4] and Jar [8, 10]. Cells were cultured in 25 cm^2 Falcon plastic flasks, in 10 ml of medium 4510 (containing 40% BSS, 50% MB 752/1 Waymouth's medium, and 10% human placental cord serum), as previously described [4], except for one set of experiments in which no serum at all was added to the medium. Cell counts were determined as reported earlier [6].

Stock solutions of MTX (methotrexate sodium, Lederle Laboratories, Chicago) were made up in BSS to concentrations ten times in excess of that desired in the incubation. MTX treatment was initiated 2 to 4 days after subculture by adding 1 volume of the stock MTX solution for every 9 volumes of medium 4510. BSS instead of MTX was added to control cultures of the same age. The medium (and either MTX or BSS) was replenished every 24 hr. At various times following medium change, flasks were used for incubation with radioactive precursors; for all flasks receiving MTX, control flasks of the same age and time after medium change, but which received no drug, were also used.

All isotopically-labeled substrates were purchased from International Chemical and Nuclear Corp., Waltham, Massachusetts. Prior to incubation with labeled precursors, the medium was removed from each flask; 2·0 ml of the same medium was readmitted to its original flask, thus providing sufficient fluid to cover completely the cell sheet on the bottom of the flask. The flask was incubated at 37°C for at least 15 min to establish temperature equilibrium before addition of the isotope. Incubation at 37°C for 30 min with either deoxyuridine-6-^{3}H (13 Ci/m-mole), L-leucine-4, 5-^{3}H (29·8 Ci/m-mole), uridine-5-^{3}H (21·7 Ci/m-mole), and/or thymidine-2-^{14}C (58·9 mCi/m-mole) was then carried out after adding the desired labeled substrate in 0·10 ml BSS. Incubations contained approximately 2·0 μCi of ^{3}H and/or 0·10 μCi of ^{14}C per ml of reaction volume. Incubations were terminated by adding a large excess of non-radioactive precursor at 0°C. The cell sheet was rinsed twice with water and stored frozen. Briefly, for measurement of radioactivity, the cells were disrupted in 5 ml water, either by freeze-thawing or sonic oscillation. Macromolecules were precipitated by adding 0·25 ml of the disrupted cell suspension into 10% TCA in a filter disc apparatus, and suction was then applied. The glass fiber disc containing the precipitate was washed successively with 10% TCA, ethanol–ether (1 : 1), then ether, and air-dried, prior to counting in a liquid scintillation spectrometer. Details of this technique have been described elsewhere [6]. In experiments where both isotopes were measured simultaneously, the windows of the liquid scintillation spectrometer were adjusted to allow a 25% overlap of ^{14}C counts in the tritium channel, and less than 0·3% overlap of tritium counts into the ^{14}C channel. Chemical estimation of DNA and protein was performed by the methods described by Hussa *et al.* [6].

For incubations with D-glucose-6-^{3}H (8·65 Ci/m-mole), the medium was removed from each flask and discarded. The cells were rinsed for 15 sec at 37° with 4510 medium to insure a constant specific activity of labeled glucose in all flasks; the rinse was discarded. Two ml of 4510 medium containing 10 μCi glucose-6-^{3}H/ml was then added at 37° to the cells to start the incubation, which was continued for 30 min. For these experiments the radioactivity in the lipid, RNA, DNA, and protein fractions was estimated by the method of Rowland [13], as were the amounts of RNA, DNA, and protein.

RESULTS

Incubation of BeWo cells for more than 1 day with either 0·10 or 10 μM MTX resulted in a significant ($p < 0·05$) decrease in cell number when compared to untreated cultures of the same age (Fig. 1). Increasing the concentration of the drug to 1000 μM gave similar results:

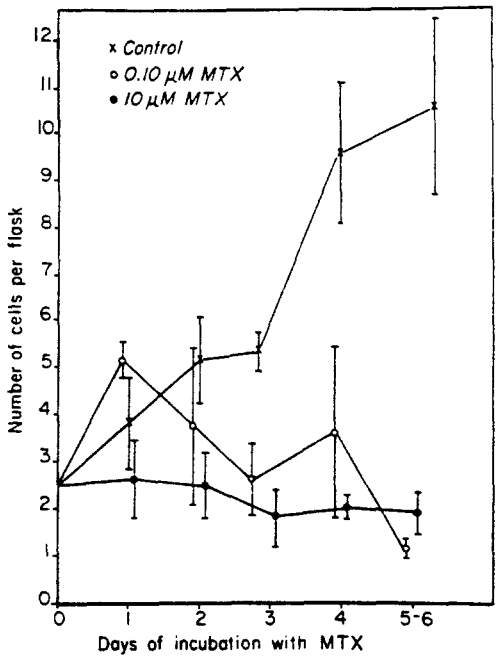

Fig. 1. Effect of MTX on cell population (in millions) in cultured human choriocarcinoma cells (BeWo line). Daily addition of MTX was initiated 2 to 4 days after subculture. Vertical lines indicate S.E.M. of 3 or more determinations. Other details are given in "Materials and Methods".

1·55 ± 0·89 million cells remained in the flask after 6 days of MTX treatment. It is noteworthy that quantitatively the same effect was produced with all doses of MTX studied, from 0·10 to 1000 μM, and that instead of all the cells eventually being killed by MTX in the flask, the population did not change significantly after initiation of drug treatment.

The same results were also found for the Jar cell line, with cell counts measured after 2 and 5 days of treatment yielding 2·08 ± 1·04, 8·40 ± 1·23 million cells (control); 0·72 ± 0·02, 1·05 ± 0·34 ($p < 0·05$) million cells (10 μM MTX); and 0·75 ± 0·09, 0·44 ± 0·15 ($p < 0·05$) million cells (1000 μM MTX), respectively. This trend was also observed when the same study was performed on a BeWo clonal line (R. A. Pattillo, unpublished data).

In addition to the decrease in cell population caused by MTX there was a gradual, dose-dependent lowering of the amount of protein and DNA in each culture flask (Table 1). Although both parameters were reduced by MTX, the decrease in protein per flask was less than that in DNA per flask, resulting in a

Table 1. Effect of MTX on protein and DNA content in human choriocarcinoma cells in culture (BeWo line)

Parameter	Dose of MTX (μM)	Days of treatment with MTX		
		1–2	3–4	5–7
Protein per flask (mg)	Control	2·53 ± 0·30	3·49 ± 0·42	4·59 ± 1·14
	0·10	3·16 ± 1·29	1·67	2·38 ± 1·13
	10	3·02 ± 0·50	2·80 ± 0·56	1·62 ± 0·31*
	1000	2·18 ± 0·05	1·57 ± 0·34*	0·25 ± 0·06*
DNA per flask (μg)	Control	197 ± 29	273 ± 41	424 ± 96
	0·10	159 ± 69	75	94 ± 40*
	10	145 ± 35	174 ± 40	68 ± 17*
	1000	136 ± 6	112 ± 5*	—*†
Cells per flask (millions)	Control	4·50 ± 0·78	7·50 ± 1·36	10·6 ± 1·88
	0·10	4·33 ± 0·25	3·20	1·12 ± 0·15*
	10	2·57 ± 0·18*	1·90 ± 0·59*	1·87 ± 0·41*
Protein per DNA (g/g)	Control	12·8 ± 1·2	12·8 ± 0·7	10·8 ± 1·7
	0·10	19·9 ± 1·8*	22·3	25·3 ± 5·5*
	10	20·8 ± 1·7*	16·1 ± 1·1*	23·8 ± 4·0*
	1000	16·0 ± 0·40*	14·0 ± 3·4	—*†
Protein per cell (pg)	Control	562 ± 91	465 ± 69	433 ± 79
	0·10	730 ± 297	522	2125 ± 1392
	10	1175 ± 208*	1474 ± 458*	866 ± 270
DNA per cell (pg)	Control	43·8 ± 4·5	36·4 ± 5·8	40·0 ± 6·5
	0·10	36·7 ± 15·9	23·4	83·9 ± 15·8*
	10	56·4 ± 13·9	91·6 ± 28·2	36·4 ± 13·4

Daily addition of MTX was initiated 2 to 4 days after subculture. The means ± S.E.M. for 3 or more determinations are given. Numbers with no S.E.M. were from single determinations. Other details are given in "Materials and Methods".

*Differs significantly ($p < 0·05$, Student's t-test) from control that received BSS instead of MTX.

†Not detectable.

significant increase in the protein: DNA ratio. Similarly, the protein content per cell was increased by MTX. Although the DNA content per cell was generally (but not always) greater in drug-treated cultures than in controls (Table 1), this increase was seldom significant. Unpublished observations using phase-contrast microscopy in our laboratory indicate that the cell size is larger in MTX-treated cells than in untreated controls, primarily due to an increase in cytoplasmic volume.

In Figs. 2 and 3 the results are plotted relative to the 24 hr control in order to realistically

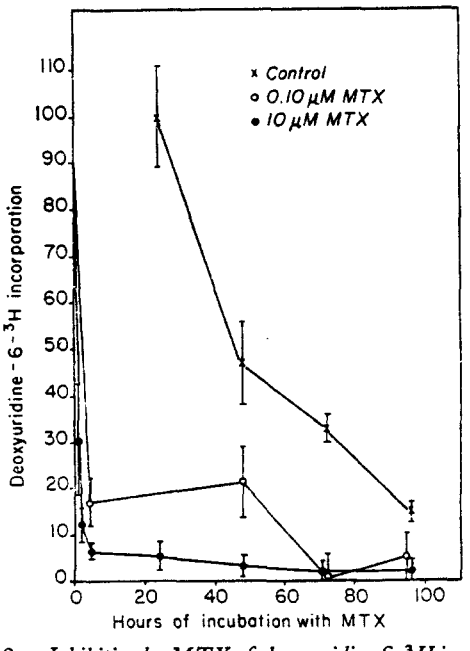

Fig. 2. Inhibition by MTX of deoxyuridine-6-³H incorporation into TCA precipitate in cultured human choriocarcinoma cells (BeWo line). After the indicated incubation period with MTX (Abscissa), deoxyuridine-6-³H was added to the culture medium (2·0 μCi/ml) and incubation was continued for 30 min at 37°. Incorporation into TCA precipitate was determined by the filter disc method [6]. Results were calculated as cpm/g protein. The values plotted (Ordinate) represent the mean ± S.E.M. of 3 or more determinations, expressed as percent of control at 24 hr (4 × 10⁶ ± 0·44 × 10⁶ cpm/g protein), which was taken as 100%. Other details are given in "Materials and Methods".

depict the decrease in precursor incorporation into DNA that occurred with culture age, as previously reported [5]. The declining rate of DNA synthesis with age in the control cultures is probably a result of the cells becoming confluent. Therefore, since the population never increases after addition of MTX to the cultures (Fig. 1), it is more appropriate to compare incorporation rates in MTX-treated cultures with rates in control flasks in which the cells have not yet attained confluency.

MTX rapidly inhibited in a dose-dependent manner the incorporation of ³H-labeled deoxyuridine into DNA in malignant trophoblastic cells (Fig. 2). After 5 hr of treatment with either 0·10 or 10 μM MTX, deoxyuridine-³H incorporation into the TCA precipitate was decreased by 83 and 93% respectively, compared to control. After 3 days of treatment virtually no DNA synthesis from ³H-labeled deoxyuridine could be observed.

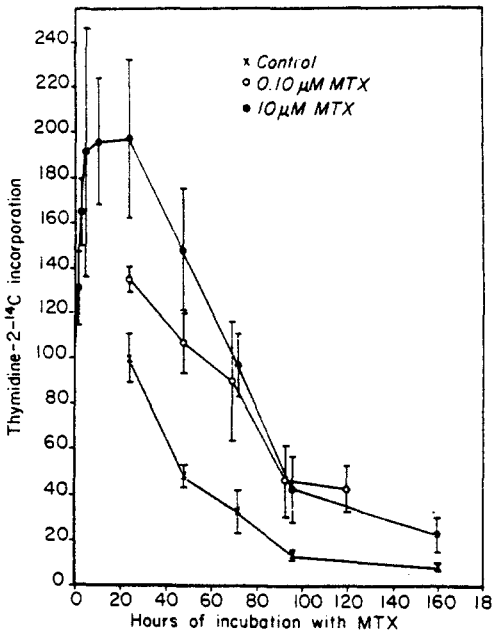

Fig. 3. Enhancement by MTX of thymidine-2-¹⁴C incorporation into TCA precipitate in cultured human choriocarcinoma cells (BeWo line). After the indicated incubation period with MTX, thymidine-2-¹⁴C was added to the culture medium (0·10 μCi/ml), and incubation was continued for 30 min. Each point represents the mean ± S.E.M. of 3 or more determinations, expressed as percent of control at 24 hr (5·6 × 10⁶ ± 0·61 × 10⁶ cpm/g protein), which was taken as 100%. Other details are the same as described in Fig. 2.

By contrast, MTX enhanced thymidine-2-¹⁴C incorporation into the TCA precipitate within 3 hr of treatment (Fig. 3). For as long as 7 days in the presence of either 0·10 or 10 μM MTX, thymidine-2-¹⁴C incorporation was consistently greater in drug-treated cells than in controls of the same age. However, the rate of incorporation in drug-treated cells after 4 to 7 days of incubation with MTX was significantly *lower* than in control cells given BSS for only 24 hr (Fig. 3).

Similar experiments were performed in which trophoblastic cells were incubated with either leucine-³H or uridine-³H after as many as 3 days of treatment with 0·10, 10, or 1000 μM MTX, and the radioactivity in the TCA precipitate was measured. In such cultures the synthesis of both protein and RNA did not differ significantly from that in control cultures given BSS instead of MTX in the daily medium (results not shown). For example, when BeWo cells were incubated for 6, 10, 24, 48, or 72 hr

with 10 μM MTX, the rate of incorporation of uridine-^{3}H into RNA (% of control, protein basis) was 123±26, 94±40, 126±16, 90±2 ($p < 0.05$), and 116±17; the corresponding rate of incorporation of leucine-^{3}H into protein was 129±16, 84±13, 122±17, 103±12, and 101±9.

Incubation of malignant trophoblastic cells of the Jar line with MTX gave results that did not differ significantly from the above when incorporation of deoxyuridine-^{3}H, thymidine-^{14}C, leucine-^{3}H, or uridine-^{3}H into the TCA precipitate was measured (results not shown).

An attempt was made to determine if MTX could affect incorporation of glucose-^{3}H into macromolecules in the trophoblastic cell to the extent that it altered the incorporation of either deoxyuridine-^{3}H or thymidine-^{14}C into macromolecules (Table 2). Treatment of the cells for at least 5 days with 10 μM MTX produced no striking changes in incorporation of glucose-^{3}H into lipid, RNA, DNA, or protein when compared to controls. On a protein basis a modest but significant inhibition of incorporation into RNA, DNA, and protein was observed. However, on a DNA basis the rates of incorporation into all types of macromolecules were never significantly less than control. These results are in accord with the increased protein: DNA ratio caused by MTX (Table 1).

The possibility was also considered that addition of 10% serum to the daily culture medium might provide enough tetrahydrofolic acid or other substances to alter the effects of MTX. Removal of the serum from the daily medium for 2 days did not markedly alter the inhibition of deoxyuridine-^{3}H incorporation into DNA, the increase in thymidine-^{14}C incorporation into DNA, or the relative decrease in cell population caused by 0·10 or 10 μM MTX. (R. O. Hussa, unpublished observations). Indeed, if a significant amount of folate derivatives were provided in the complete medium, one would anticipate less than complete inhibition of deoxyuridine-^{3}H incorporation into DNA.

DISCUSSION

The work of many investigators suggests that the major effect of MTX is inhibition of the enzyme dihydrofolate reductase. This prevents the reduction of dihydrofolic acid to tetrahydrofolic acid, needed for the formation of $N^{5,10}$-methylenetetrahydrofolic acid. The latter cofactor is required in substrate amounts by the enzyme thymidylate synthetase for methylation at the 5 position of deoxyuridylic acid to form thymidylic acid, a pyrimidine essential for DNA synthesis. Although tetrahydrofolic acid is also required as a C_1 carrier in 2 reactions of purine synthesis, neither of these reactions is oxidative in nature, thereby obviating direct involvement of dihydrofolate reductase in purine biosynthetic pathways. The lack of deoxyuridylic acid conversion results in a reduction in the cellular levels of thymidylic acid. Consequently, DNA cannot be adequately synthesized, preventing cell replication (For details, see refs. 14–18).

The present results are compatible with the interpretation that the malignant trophoblastic cells are sensitive to MTX *in vitro* as a result of inhibition of dihydrofolate reductase by the

Table 2. Effect of 10 μM MTX on synthesis of macromolecules from D-*glucose-6-*^{3}*H in human choriocarcinoma cells in culture (BeWo line)*

Days of MTX treatment	Protein basis				DNA basis			
	Lipid	RNA	DNA	Protein	Lipid	RNA	DNA	Protein
1	109 ± 14	81·3 ± 8·9	92·3 ± 3·8	109 ± 13	139 ± 24	102 ± 7·2	117 ± 0·6*	136 ± 13
2	82·0 ± 15·0	68·0 ± 3·0*	67·0 ± 1·0*	75·5 ± 0·5*	162 ± 38	135 ± 14	134 ± 10	151 ± 11*
3	87·7 ± 11·0	54·0 ± 6·9*	65·7 ± 5·8*	83·7 ± 7·2	117 ± 18	71·7 ± 11·5	85·7 ± 5·5	109 ± 5·0
5	148 ± 16	73·5 ± 4·5*	49·0 ± 1·0*	72·0 ± 4·0*	330 ± 85	173 ± 35	120 ± 40	190 ± 45

Results were calculated as percent of control that received BSS instead of MTX in culture medium, and are expressed as mean ± S.E.M. from 3 determinations.

*Differs significantly ($p < 0.05$, Student's t test) from control.

antimetabolite. Preliminary studies in this laboratory have demonstrated rapid and complete inhibition of the enzyme by 0·10 μM MTX in cell-free preparations from malignant trophoblastic cells. This causes a rapid decrease in deoxyuridine incorporation into DNA (Fig. 2), concomitant with an early compensatory increase in thymidine incorporation into DNA (Fig. 3). Apparently the salvage pathway (i.e. thymidine kinase) cannot fill the cells' demands for thymidylic acid required for normal production of DNA. The trophoblastic cells, thus hindered in replicative capacity, become abnormally large as their processes for synthesizing RNA, protein, and lipid operate at normal rates (Table 2). Consequently, the cell population does not increase (Fig. 1), but the cell size increases as measured by protein content (Table 1) and light microscopy. After 4 days (Fig. 3) the capacity of the cells to incorporate thymidine into DNA decreases below the level observed in the 1-day control cultures, reflecting severe cell toxicity.

The eventual decline in thymidine utilization suggests a decreasing ability of the existing cells to synthesize DNA, rather than a constant turnover rate of cells in a healthy population. In the latter case, one would expect the cells to maintain their capacity for DNA synthesis, particularly in the absence of confluent growth. In agreement with this interpretation, we have been unable to successfully subculture malignant trophoblastic cells that were previously incubated for several days on concentrations of MTX greater than 0·10 μM. However, indefinite maintenance can be achieved with cells chronically maintained on medium containing 0·01 μM MTX.

The relative inability of MTX to inhibit incorporation of leucine-^{3}H or uridine-^{3}H into the TCA precipitate, or of glucose-^{3}H into RNA, DNA, protein, or lipids, further suggests that the drug's inhibitory effect is relatively specific for the thymidylate synthetase step in pyrimidine biosynthesis.

Hreshchyshyn and co-workers [11, 12] found that MTX decreased tumor volume, cell population, and number of cells in mitosis of Wo human choriocarcinoma and benign trophoblast in the hamster cheek pouch. Our results with the BeWo cell line *in vitro* are in excellent agreement with their studies. This correlation is noteworthy because the *in vitro* BeWo cell line was derived 6 years ago from the Wo choriocarcinoma [4]. As discussed earlier, both the Wo choriocarcinoma and the Jar cells were obtained from patients whose tumors no longer responded to MTX [8–10], suggesting that the clinical resistance to the inhibitory effect of MTX is *not* associated with resistance of the primary pathway affected by the drug. Additional studies to clarify this point are presently in progress in this laboratory.

Our results can also be compared with those from other *in vitro* model systems, although rigorous comparisons and speculations are not warranted at the present time. In accordance with our findings, *in vitro* studies on drug-sensitive lines of mouse leukemia cells [18, 19] demonstrated inhibition of deoxyuridine incorpora tion into DNA, in contrast to the lack of inhibition in a MTX-resistant line [18].

Also in agreement with the present findings on thymidine incorporation, Pegoraro and Benzio [20] observed more than a threefold increase in thymidine-^{3}H incorporation into DNA when human lymphocytes were incubated for 48 hr with phytohemagglutinin and 10 μM MTX, concomitant with a 50% inhibition of DNA synthesis from $H_3{}^{32}PO_4$. Roberts and Wodinsky reported an increase in thymidine incorporation into DNA following administration of MTX to sensitive mouse leukemic cells, but no change when MTX-resistant cells were studied [18]. On the other hand, Hryniuk *et al.* observed *inhibition* of thymidine-^{3}H incorporation into DNA by 1 μM MTX in cells from logarithmically growing cultures of mouse leukemia cells, and little effect in cells from resting cultures [19]. Thirdly, other workers have also observed little or no effect of MTX on biosynthetic pathways not involving thymidylate synthetase [14, 16, 20].

This is the first report of the effects of MTX on cell growth and incorporation of precursors into macromolecules in human choriocarcinoma maintained in continuous cell culture. Using this model system we believe meaningful information can be obtained on the development of drug resistance in the malignant trophoblast. To this end, the recent development of two malignant trophoblastic cell lines derived from a single patient, before and after the development of MTX resistance *in vivo* [8], will be of significant value in the continued effort to identify the specific mechanism of this drug's actions as well as those alternate pathways permitting the development of resistance.

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Time-Dose Relationships in Combined Bleomycin Treatment and Radiotherapy

SVEND JUUL JØRGENSEN

The Fibiger Laboratory, Lundtoftevej* 5–7, 2800 *Kgs. Lyngby, Denmark*

Abstract—*The biological effect of bleomycin resembles that of X-ray irradiation indicating that the combination of bleomycin and radiotherapy may have a synergistic effect.*

In cyclic bleomycin treatment of a murine carcinoma it was found that intermittent bleomycin treatment and radiotherapy results in a simple addition of the therapeutic effects of the two different treatments. Simultaneous treatment, however, results in a synergistic intensifying of the therapeutic effect.

The toxicity of bleomycin was found to be unaltered by the regional application of X-ray irradiation.

IN A PREVIOUS report on experimental bleomycin treatment it was stated that cyclic treatment offered a better therapeutic index than treatment with the drug given daily or twice a week [1]. The improvement of the therapeutic index was provided by a marked reduction of the toxicity following the cyclic treatment. On the other hand it was not found possible to raise the efficiency curve to a higher level by the cyclic administration of the drug.

Other investigators have suggested that the combination of bleomycin treatment with radiotherapy would give an improved therapeutic effect as bleomycin causes a scission of DNA and inhibits DNA synthesis as well as DNA repair [2, 3]. However, no experimental studies on combined bleomycin treatment and radiotherapy have been reported until now.

MATERIAL AND METHODS

The effect of bleomycin and x-irradiation was tested on a murine epidermoid carcinoma transplanted into 5 to 9-week old male and female mice. The strain of experimental animals (STAC3F_1 mice) and bleomycin were identical with those used in the previous study [1]. The test tumour was also the same but in its 55th to 59th passage of transplantation. The tumour was transplanted by the intramuscular injection in the right thigh of 0·1 ml of a tumour suspension prepared by mincing with sharp scissors. The therapeutic efficiency was evaluated by the reduction of tumour weight as compared to the untreated control group. The tumour weight was calculated as the difference between the weight of the right thigh with tumour and the normal left thigh. Radiotherapy was given as one weekly irradiation of 300 rad of the tumour bearing part of the body (200 KV; 1·00 mA; n-filter HVL 1, 3 mm Cu; dose rate: 34 rad/min; distance 590 mm). The rest of the animal was shielded by 2 mm Pb. Bleomycin was given according to the cyclic schedule [1] previously described (6 injections/48 hr/week in 3 weeks). Radiotherapy was given either *simultaneously* with the individual cycles of bleomycin treatment between the 1st and 2nd injection or *intermittently* i.e. in the middle of the intervals between the bleomycin cycles (Fig. 1).

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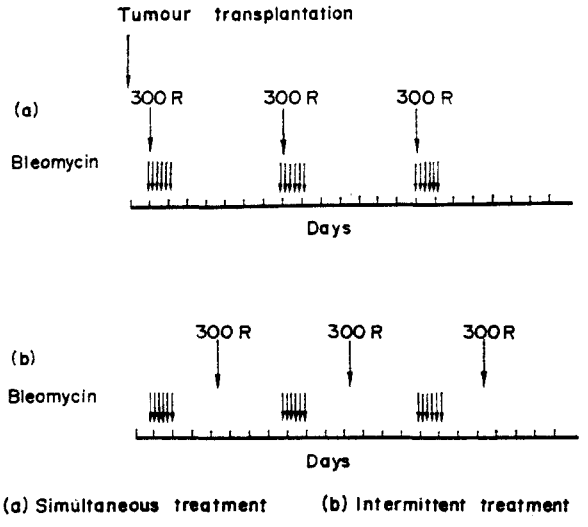

Fig. 1. Time relationships in sumultaneous and intermittent bleomycin treatment and radiotherapy.

Statistical methods

Each tumour weight in the treated group was compared to the weight of a non-treated control tumour chosen at random. The tumour reduction of each group was estimated as the mean of the figures for all mice in the group. The dosage–response curves established in this way can be transformed to linear curves as described by Westerfield [4]. The figures were subjected to regression analysis and the statistical significance was calculated.

RESULTS

The tumour was found to be sensitive to irradiation. Three hundred rad/week in 3 weeks resulted in a 76·3% reduction of tumour growth. The efficiency curve for the cyclic bleomycin treatment was previously established [1]. This curve is shown in Fig. 2 together with the lethality curve and the efficiency curves established in the present study.

The toxicity of bleomycin in combination with radiotherapy was tested in a lethality test. Radiotherapy was given simultaneously as regional irradiation using the same dose and region as in the therapeutic series (300 rad/week in 3 weeks). The test was performed as a LD_{50} test. The results of this test are seen in Table 1. The figures were subjected to probit analysis. By this method the LD_{50} in this study was found to be 338 mg/kg while the LD_{50} in bleomycin treatment alone was found to be 335 mg/kg. This means that the toxicity of bleomycin is unchanged in the combination therapy. Therefore, in Fig. 2 the lethality curve previously established [1] is shown together with the efficiency curves. The results obtained by the simultaneous bleomycin treatment and radiotherapy are seen in Table 2, while those obtained by the intermittent treatment are shown in Table 3.

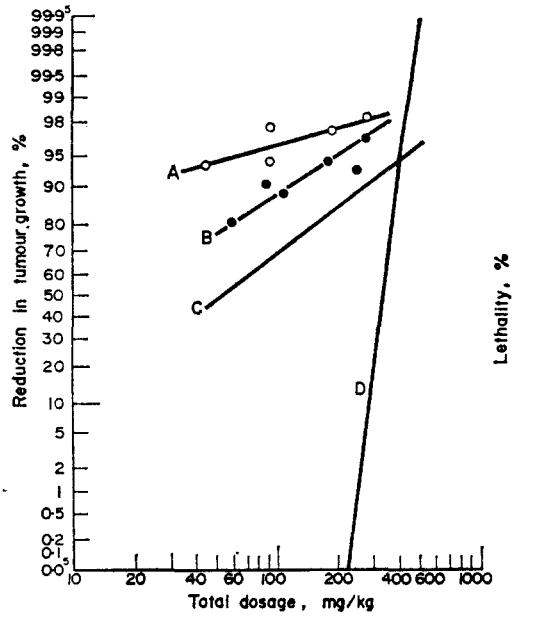

Fig. 2. Lethality curve and efficiency curves in probit system. Curve A: dosage–response curve for simultaneous bleomycin and radiotherapy. Curve B: dosage–response curve for intermittent treatment. Curve C: dosage–response curve for cyclic bleomycin treatment. Curve D: lethality curve for cyclic bleomycin treatment.

From Fig. 2 it is seen that the inhibition of tumour growth obtained by the combined treatment was higher than by bleomycin treatment alone. Furthermore, it was found that the results obtained by the simultaneous treatment were better than by the intermittent treatment. The curve for the intermittent treatment was almost parallel to the curve for bleomycin treatment alone, but displaced to a higher level. This difference is statistically significant ($p < 0{\cdot}01$).

Table 1. Simultaneous bleomycin treatment and radiotherapy (300 rad/week). Lethality test.

Bleomycin Total dose (mg/kg)	No. of mice	Lethality
291·0	9	4/9
305·0	9	4/9
355·0	10	5/10
404·0	10	6/10

The curve for the simultaneous treatment differs from the one for the intermittent treatment by having a different slope and by being at a higher level. The difference is statistically

Table 2. Simultaneous radiotherapy and bleomycin treatment*

	Total dose bleomycin (mg/kg)	No. of mice	Weight of mice (g ± S.E.M.)		Weight of tumour (g ± S.E.M.)	% Reduction of tumour growth (95% confidence limits)
			Before treatment	After treatment		
Control A		5	23·0 ± 0·8	31·2 ± 1·2	7·07 ± 0·52	
Group A	94·7	5	19·0 ± 0·8	18·5 ± 0·5	0·29 ± 0·10	97·56 (95·98–98·53)
Control B		5	18·0 ± 0·4	26·4 ± 0·4	8·05 ± 0·31	
Group B1	45·0	5	20·0 ± 0·0	18·4 ± 0·5	0·54 ± 0·03	93·34 (92·94–93·72)
Group B2	92·7	5	19·4 ± 0·4	19·8 ± 0·4	0·44 ± 0·05	94·66 (93·68–95·50)
Group B3	189·0	5	19·0 ± 0·4	15·4 ± 0·4	0·34 ± 0·13	96·63 (95·24–97·63)
Control C		5	23·6 ± 0·6	30·8 ± 0·5	5·93 ± 0·38	
Group C	238·9	5	22·6 ± 1·0	18·8 ± 0·9	0·17 ± 0·06	98·18 (96·44–99·08)

*A total dose of 900 rad was given. 5 mice receiving X-irradiation alone without bleomycin treatment showed a reduction of tumour weight from 8·05 ± 0·32 g to 1·74 ± 0·4 g and 4 mice a reduction from 8·96 ± 0·65 g to 2·31 ± 0·17 g. Mean reduction of tumour growth 76·3%.

Table 3. Intermittent radiotherapy and bleomycin treatment*

	Total dose bleomycin (mg/kg)	No. of mice	Weight of mice (g ± S.E.M.)		Weight of tumour (g ± S.E.M.)	% Reduction of tumour growth (95% confidence limits)
			Before treatment	After treatment		
Control		5	20·4 ± 0·2	34·8 ± 0·6	8·95 ± 0·65	
Group 1	57·5	5	15·6 ± 0·4	19·8 ± 0·4	1·73 ± 0·26	81·29 (78·70–83·63)
Group 2	87·1	5	20·6 ± 0·2	23·4 ± 0·4	0·83 ± 0·15	91·32 (88·76–93·33)
Group 3	108·3	5	16·6 ± 0·5	25·4 ± 0·2	1·13 ± 0·18	87·78 (85·61–89·66)
Group 4	189·0	5	19·0 ± 0·8	19·2 ± 1·1	0·50 ± 0·12	94·70 (93·42–95·74)
Group 5	236·8	5	15·2 ± 0·5	17·2 ± 0·5	0·77 ± 0·12	92·25 (90·76–93·51)
Group 6	264·6	5	20·4 ± 0·7	19·8 ± 1·1	0·30 ± 0·04	96·70 (96·05–97·24)

*See footnote in Table 2.

significant ($p < 0·01$). Thus the simultaneous bleomycin treatment and radiotherapy offers significantly better results than combination thereapy given intermittently.

DISCUSSION

Terasima *et al.* [3] found that bleomycin causes breaks of DNA in cultured cells and that this effect is reversible. After removing bleomycin from the culture, DNA was found to rejoin. Quite similar conditions were found in cells exposed to X-ray irradiation. Also the changes in sensitivity during the cell cycle was found to be identical for bleomycin and radiotherapy [2, 5]. As the biological effects of bleomycin seems to be similar to the effects of radiotherapy, a synergistic effect may be expected by combining the two treatments.

In the present study, the combination therapy was given after two different schedules. Following the intermittent schedule a simple addition was to be expected. Because of the time interval between the bleomycin and radiotherapy the tumour might be expected to recover after bleomycin treatment before it is exposed to irradiation. In the same way the biological changes in the tumour caused by the irradiation probably will have disappeared before a new bleomycin cycle is started.

Consequently, the two different treatments are not expected to interfere with each other. This theory is confirmed by the findings in this study. The efficiency curve for the intermittent treatment was found to be parallel to the curve for bleomycin treatment alone, indicating that there is a simple additional effect of the combination therapy. Contrary to this the efficiency curve for the simultaneous treatment was found to be significantly different from the two other curves. The very high efficiency at small doses indicates that the two treatments are reciprocally intensifying one another. As stated, the toxicity of bleomycin was found to be unaltered by the regional irradiation. This does not necessarily indicate that the side effects of bleomycin might not be increased by the combination therapy in all cases. Thus, pulmonary fibrosis could be expected to appear more frequently by combining radiotherapy and bleomycin treatment of for instance bronchogenous carcinomas or mediastinal lymphomas.

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Effect of Chlorite-Oxidized Oxyamylose on Moloney Sarcoma Virus-Induced Tumor Formation in Mice*

E. DE CLERCQ† and P. DE SOMER

Rega Institute for Medical Research, University of Leuven, Leuven, Belgium

Abstract—*Chlorite-oxidized oxyamylose (COAM) effectively inhibited tumor growth in NMRI mice infected neonatally with the Moloney strain of murine sarcoma virus (M-MSV). This protective effect was most pronounced with single doses of the compound administered shortly before or after virus challenge. At the appropriate dosage level, COAM was also effective when it was administered as a single dose at 7 or 13 days after the virus or when it was injected in repeated doses on alternate days from 7 till 19 days or from 13 till 23 days after virus challenge. COAM reduced both tumor incidence and death due to tumor growth. However, with the treatment regimens used, it did not cause a regression of tumors which had already appeared at the time treatment was initiated.*

INTRODUCTION

POLYACETAL carboxylic acids belong to a new group of antiviral compounds whose interferon-inducing capacity and antiviral activity has been described recently [1, 2]. Chlorite-oxidized oxyamylose (COAM), the most extensively studied congener of the group, appears to protect mice against several virus infections including mengo, vaccinia, Semliki Forest, influenza APR 8, foot and mouth disease and vesicular stomatitis virus [1–6]. Although COAM stimulates the production of interferon in the blood stream [1], non-interferon mediated mechanisms such as activation of reticuloendothelial cells with consequent trapping of virus in these activated cells have been proposed to account for the *in vivo* antiviral activity of COAM [3, 4].

So far, COAM has only been tested in one tumor model system: spontaneous mammary carcinoma in C_3H mice [7]; neonatal administration of the compound resulted in an inhibition of the formation of these tumors.

In this report we describe the antitumor effect of COAM in mice neonatally infected with the Moloney strain of murine sarcoma virus. This experimental tumor model has been used previously to assess the antitumor activity of interferon [8–11] and interferon inducers such as polyriboinosinic acid-polyribocytidylic acid [poly (rI). poly (rC)] [10–15].

MATERIAL AND METHODS

Animals

Randomly bred NMRI mice used in these experiments were obtained from the Animal Production Section of the University of Leuven. Newborn mice were housed at 10 mice per cage with one mother.

Murine sarcoma virus

A stock of murine sarcoma virus (Moloney strain: M-MSV) was prepared as described previously [11]. The stock contained a tumor-inducing dose 50 (TID_{50}) of 10^3 per 0·025 ml when titered in 2-day old NMRI mice. The

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virus concentrate was used at 2 dilutions: $10^{-2.3}$ (Figs. 1 and 2) and $10^{-2.6}$ (Fig. 3). The virus dilutions were prepared in a solution containing 0·5 M sucrose and 0·00075 M tris-HCl, pH 7·2; 0·025 ml of either dilution was injected intramuscularly (right thigh) at approximately 48 hr after birth.

Chlorite-oxidized oxyamylose (COAM)

COAM was synthesized by Dr. P. Claes (Rega Institute, University of Leuven) as described previously [1]. The compound was dissolved in sterile physiological saline and administered intraperitoneally, at the time and dosage levels indicated below.

Assessment of antitumor activity

The antitumor activity of COAM was assessed by two parameters: tumor incidence and mortality due to tumor development. Tumor incidence and mortality were recorded daily.

RESULTS

Effect of a single dose of COAM injected 1 day before or 1 day after virus inoculation

A single dose of 0·5 mg of COAM injected 1 day before or 1 day after M-MSV challenge exerted a significant inhibitory effect on the development of MSV tumors: COAM treatment decreased the incidence and delayed

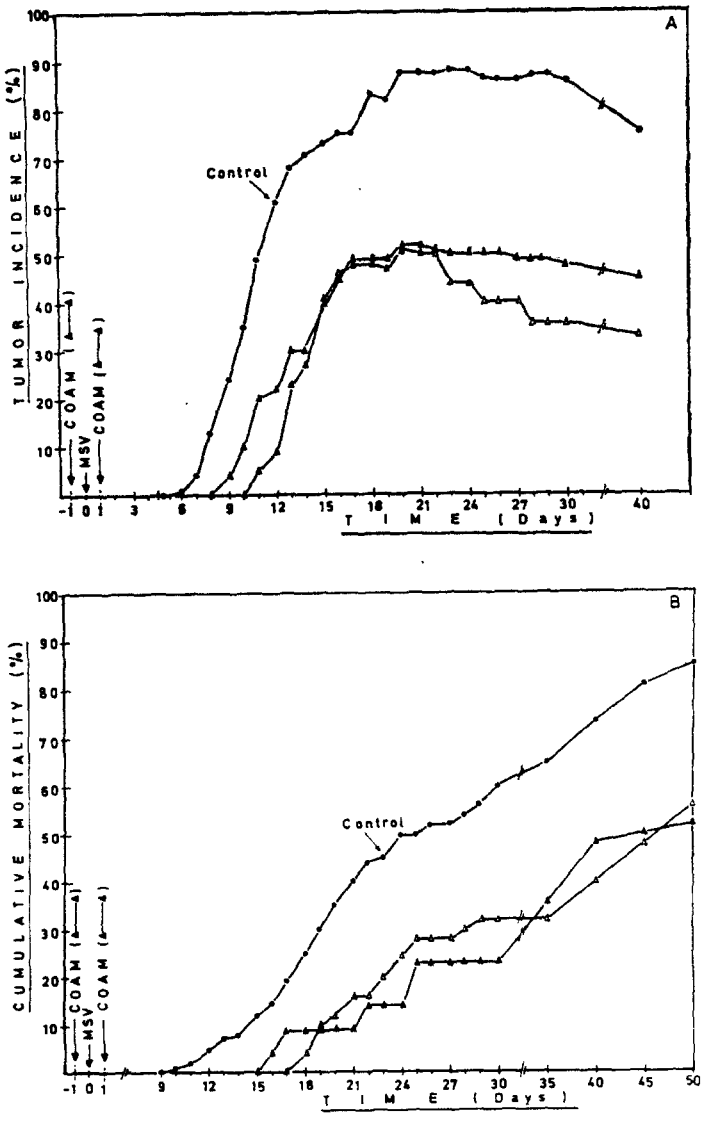

Fig. 1. Effect of a single dose of COAM (0·5 mg/mouse), injected either 1 day before or 1 day after virus challenge, on tumor incidence (A) and mortality (B) in mice infected neonatally with M-MSV ($10^{-2.3}$ dilution). Thirty mice per group (eighty mice for control group).

the appearance of tumors (Fig. 1A), and also reduced the mortality rate (Fig. 1B). There was no marked difference in the effects of single injections of COAM, given 1 day before or 1 day after virus inoculation.

Effect of a single dose of COAM injected at 7 days and of repeated doses of COAM starting at 7 days after virus inoculation

Although a single dose of 0·5 mg of COAM administered at 7 days after M-MSV challenge did not affect minor development (not shown), increasing the dose to 2·0 mg resulted in a significant reduction of tumor incidence (Fig. 2A) but did not change the death rate (Fig. 2B). Repeated doses of 2·0 mg of COAM administered at 7, 9, 11, 13, 15, 17 and 19 days after virus challenge did also decrease tumor incidence (Fig. 2A), and, in addition, brought about a slight reduction of the mortality rate (Fig. 2B).

Effect of a single dose of COAM infected at 13 days and of repeated doses of COAM starting at 13 days after virus inoculation

The effects of a single dose of COAM administered at 13 days and of repeated doses of COAM starting at 13 days after virus challenge were assessed in mice receiving a

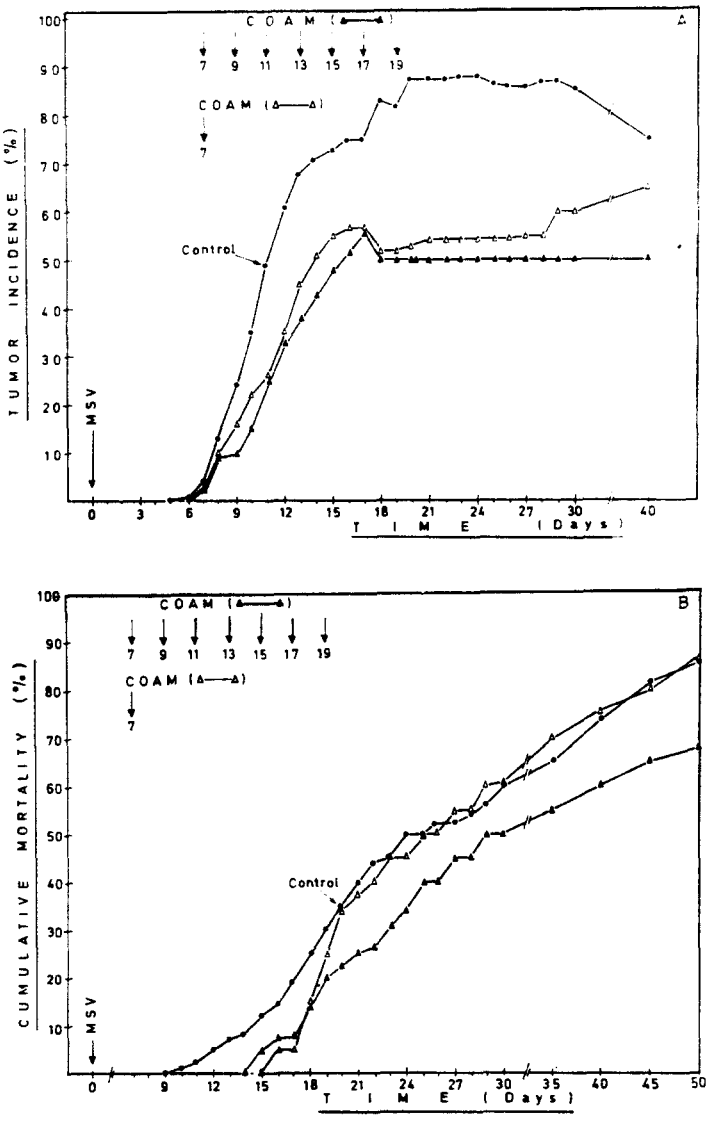

Fig. 2. Effect of a single dose of COAM (2 mg/mouse), injected at 7 days after virus challenge, and of repeated doses of COAM (2 mg/mouse), injected at 7, 9, 11, 13, 15 and 17 days after virus challenge, on tumor incidence (A) and mortality (B) in mice infected neonatally with M-MSV ($10^{-2.3}$ dilution). Thirty mice per group (eighty mice for control group).

$10^{-2.6}$ (instead of $10^{-2.3}$) dilution of the M-MSV stock. A single dose of 1·0 mg of COAM administered at 13 days after virus challenge did not markedly influence tumor development (not shown). A single dose of 4·0 mg of COAM administered at 13 days decreased tumor incidence (Fig. 3A) but did not decrease mortality (Fig. 3B). Repeated doses of 4·0 mg of COAM administered at 13, 15, 17, 19, 21 and 23 days after virus challenge decreased both the tumour incidence (Fig. 3A) and mortality (Fig. 3B). COAM reduced the development of new tumors, but did not cause regression of tumors which had already appeared at the time COAM treatment was initiated (13 days after virus challenge).

At the dosage levels used, COAM did not cause gross symptoms of toxicity. A slight inhibitory effect on the growth of the animals, comparable to the inhibitory effect obtained with the "high" dosage schedule of COAM in previous studies [7], was noted in mice receiving multiple injections of COAM from the 7th or 13th day after virus inoculation.

DISCUSSION

Polycarboxylates such as pyran (maleic anhydride/divinyl ether) copolymer, polyacrylic acid and COAM stimulate circulating interferon production in the mouse [1, 16–18]. These polymers have also been found effective

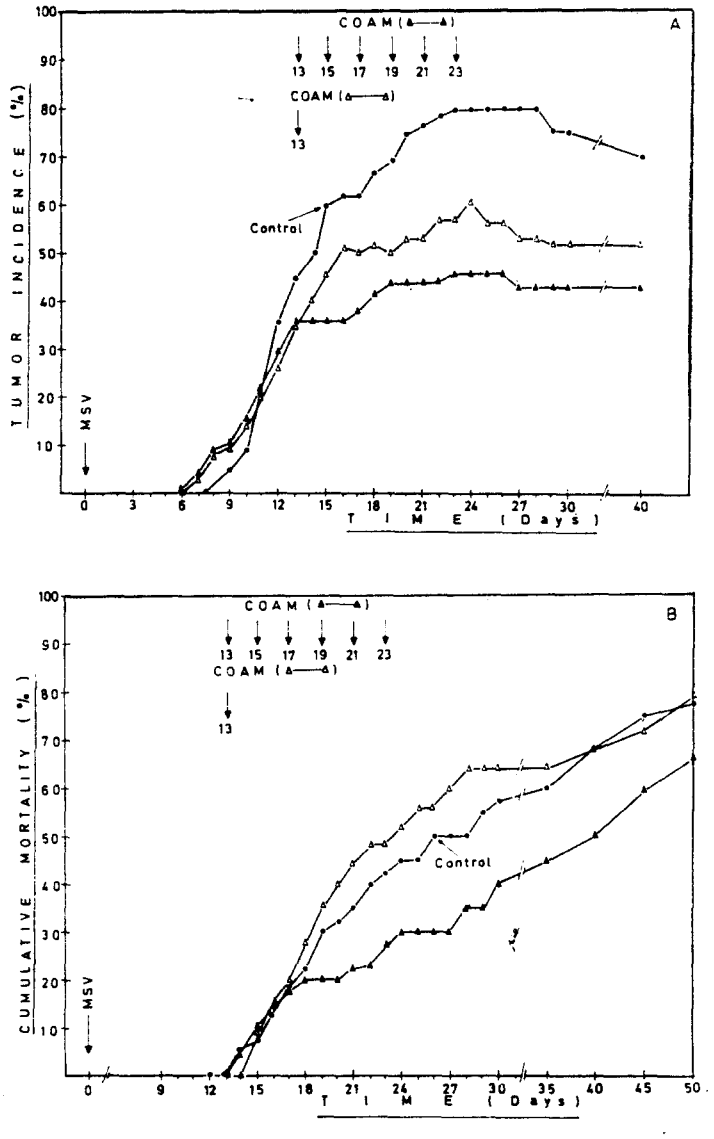

Fig. 3. Effect of a single dose of COAM (4 mg/mouse), injected at 13 days after virus challenge, and of repeated doses of COAM (4 mg/mouse), injected at 13, 15, 17, 19, 21 and 23 days after virus challenge, on tumor incidence (A) and mortality (B) in mice infected neonatally with M-MSV ($10^{-2.6}$ dilution). Thirty mice per group (60 mice for control group).

in a few tumor model systems: pyran copolymer against Friend and Rauscher leukemia in mice [19], polyacrylic acid against polyoma virus-induced tumors in mice and rats [20], and COAM against mammary carcinoma in C_3H mice [7]. Protection against Friend and Rauscher leukemia was only observed when treatment with pyran copolymer was initiated prior to virus challenge [19]. With polyacrylic acid, protection was obtained when the compound was injected as late as 2 days after virus challenge [20].

In the experimental model used in this study, COAM inhibited tumor development, even if treatment was initiated 7 or 13 days after virus challenge. COAM delayed and partially prevented the appearance of new MSV tumors but did not cause a regression of tumors which had already grown to a palpable size. The effectiveness of late treatment with COAM in mice, infected with Moloney sarcoma virus may be connected to particularities in the virus model system (i) or compound (ii) used. Using the same model, Sarma *et al.* [12] demonstrated a regression of established tumors upon treatment with poly (rI) . poly (rC); and, in mice inoculated intranasally with a lethal dose of vesicular stomatitis virus, COAM appeared to reduce the mortality rate when injected as late as 5 days after virus challenge [6].

What is the mechanism that may account for the inhibitory effect of COAM on MSV tumor development? There are different possibilities that should be considered: (i) interferon production [1]; (ii) activation of reticuloendothelial cells [4]; (iii) enhancement of antibody formation [21]; (iv) a direct chemotherapeutic effect on tumor metabolism, similar to the effects described with poly (rI) . poly (rC) [22]. The antitumor activity of a single dose of COAM injected 1 day prior to virus inoculation may be attributed to interferon production: first, COAM is capable of stimulating interferon production in mice of different age, including newborn mice (our unpublished data), and, second, interferon itself has been shown to suppress tumor development and mortality, when injected shortly before MSV challenge [11]: administered in amounts that closely mimicked the interferon blood levels induced endogenously by poly (rI) . poly (rC), exogenous interferon conferred essentially the same protection as a single dose of poly (rI) . poly (rC) administered 12 hr before virus inoculation. Whether the antitumor activity obtained with COAM injected at 1 day, 7 or 13 days after virus challenge, is also attributable to interferon production, can only be ascertained by further studies. Alternatively, the observed antitumor effects of COAM in MSV-infected mice may be accounted for by an enhanced immunological responsiveness. COAM has been shown to enhance the formation of circulating antibody: hemagglutinins in mice immunized with sheep red blood cells and hemagglutination-inhibition antibodies in mice challenged with influenza virus [21]. Billiau *et al.* [7] favored the interpretation of an accelerated immunological maturation to explain the protective effect of neonatally administered COAM against "spontaneous" mammary carcinoma in mice. Whether this mechanism might be responsible for the observed antitumor activity of COAM in MSV-infected mice, is also subject of further study.

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Shielding of Antigens and Concanavaline A Agglutination Sites by a Surface Coat of Transplantable Mouse Lymphosarcoma Cells

L. A. SMETS and J. BROEKHUYSEN-DAVIES

Department of Experimental Cytology, The Netherlands Cancer Institute, Sarphatistraat 108, *Amsterdam*

Abstract—*Agglutination with concanavaline A of mouse lymphosarcoma cells cultivated* in vitro *or propagated by serial transplanatation in mice was studied in relation to transplantability. Agglutination was high in cells cultured* in vitro *but low in cells isolated from transplantable tumors. Agglutination sites of the latter cells became exposed following proteolytic treatment or osmotic swelling. When transplantable tumors were explanted in tissue culture, agglutinability gradually reappeared depending on cell density and culture conditions. The reappearance of agglutination sites was correlated with a loss of transplantability in unirradiated mice.*

These findings have indicated that the transplantable tumor cells were covered by a coat, presumably of glycoproteins, which masked transplantation antigens as well as concanavaline A agglutination sites and that this coat got lost in tissue culture.

INTRODUCTION

IN MANY cell systems *in vitro* it has been demonstrated that normal cells have cryptic agglutination sites for plant agglutinins which become exposed on the cell surface following malignant transformation or can be unmasked by a brief proteolytic treatment [1]. Consequently, it has been postulated by Burger and Noonan [2] that normal cells contain a coat which renders agglutination sites cryptic and keeps growth under control. This theory is supported by the return to "controlled" growth of transformed cells provided with an artificial coat [2] and by the escape from growth control observed with normal cells exposed to proteolytic enzymes [3].

However, exceptions to this scheme have been reported. Gannt *et al.* [4] found no difference in agglutinability with wheat germ agglutinin between several cultures of neoplastic and normal cells. Moore and Temin [5] found no correlation between agglutinability of many normal and RNA virus transformed cells. We have studied the agglutination with concanavaline A (Con A) using mouse lymphosarcoma cells propagated either in tissue culture or by serial transplantation in mice. Evidence was obtained that a coat of (glyco) proteins shielded the agglutination sites of cells grown *in vivo*, indicating that the cryptic state of agglutination sites is not exclusive property of untransformed or normal cells. Agglutination sites of cells *in vivo* became exposed again following trypsin treatment, osmotic swelling or following explantation in tissue culture.

Transplantability of several murine tumors has been ascribed to the presence of a surface coat which reduces antigenic expression [6, 7, 8]. Therefore, we studied the cryptic state of agglutination sites with regard to transplantability. The results to be presented have indicated that the surface coat of tumor cells growing *in vivo* masked agglutination sites as

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well as transplantation antigens. Following explantation in tissue culture, these transplantation antigens became exposed again together with the aggutinatin sites.

MATERIAL AND METHODS

Cells

The cell strain MB VIA has been developed from an explant of a transplantable tumor that originated spontaneously as lymphosarcoma in a (C_{57} Black × DBA) F_1 hybrid mouse [9]. The lymphoblastic cells were propagated in modified Erlemeyer flasks in suspension culture without agitation. The culture medium consisted of Hanks' balanced salt solution (HBSS) supplemented with 0·5% lactalbumine hydrolysate, 10% newborn calfserum and antibiotics. Growth pattern and karyology were still the same as described in 1963 [10].

Syngeneic female (C_{57} Black × DBA) F_1 hybrids were used at the age of 2 to 3 months to test for the malignant potential of cultured MB VIA cells and for serial passage of tumors. The inoculum size for serial transplantation was 0·4 ml of undiluted ascites or chopped tissue in case of subcutaneous tumors. The tumors were lethal within one week. As outlined under results, a new line of transplantable tumors was started from cultured MB VIA cells. These tumor cells have been used in the experiments to be reported and were from passage numbers 20 to 40.

Preparation of cell suspensions

Suspensions of intraperitoneal or subcutaneous tumors were prepared from ascites fluid or from minced tissue. Mincing of subcutaneous tumors yielded single cell suspensions without additional treatment. Cell suspensions were diluted with Tyrode salt solution to a concentration of 10^7 cells/ml and layered on top of an equal volume of 10% Isopaque (Nyegaard & Co., Oslo; 144 mg J/ml) in ficoll buffer [11]. The gradients were run for 10 min at 2000 rev/min and tumor cells were collected from the interphase. Contaminating red cells and cellular debris sedimented to the bottom. After a second run on a ficoll-Isopaque gradient, cells were washed in buffer solution, counted with a haemocytometer and suspended in growth medium. Cell viability was assayed by mixing equal volumes of cell suspension and a 1% solution of trypan blue in buffer solution. As judged from dye exclusion, freshly prepared suspensions contained more than 90% viable cells.

Agglutination test

To test for agglutination with Con A (Calbiochem, A grade), 2×10^6 cells were washed in HBSS and resuspended in 1 ml HBSS containing Con A in the desired concentration. The suspensions were transferred to small Erlenmeyer flasks and put on a giratory shaker operated at 60 rev/min at 37°C. The suspensions were viewed at regular intervals under a binocular microscope and the time to reach maximal agglutination was recorded. Agglutination was considered maximal when virtually all cells were trapped in aggregates.

Hypotonic treatment

Swelling experiments were performed by incubation of cells in various dilutions of HBSS. Since lowering the concentrations of Ca^{2+} and Mg^{2+} was toxic to the cells, the concentrations of these ions were kept unchanged at all HBSS dilutions. The degree of swelling was measured by the increase in cell diameter. For this purpose, aliquots of the suspensions were viewed in a haemocytometer, and cell diameter was measured with a micrometer eyepiece.

Immunological techniques

To test for immunoglobulins, air-dried smears of cells were incubated with fluorescein–isothyocyanate conjugated swine anti mouse globulin antibody (Nordic Diagnostics: Sw AM/FITC) in a 1:10 dilution. Undiluted conjugate was controlled for the presence of anti-immunoglobulins by immunoelectrophoresis with mouse serum. A positive reaction for IgG, IgA and IgM was obtained. The cells were inspected with a Zeiss immunofluorescence microscope with incident light using excitation filters BG 38, BG 12 and barrier filter 50 and a high-pressure mercury lamp HBO 200. To dissociate antibodies from tumor cells, suspended cells were washed and incubated for 30 min in isotonic glycine-HCl buffer at pH 2·8 followed by 2 washings in HBSS.

Transplantability

Transplantability of MB VIA cells and of cells isolated from transplantable tumors was assayed by intraperitoneal injection of 10^6 cells suspended in 0·4 ml of saline. Transplantability was expressed as the surviving time of recipient mice. Animals surviving for 60 or more days were scored as tumor-free, the challenging cells accordingly as non-transplantable. Partial immunosuppression in ani-

mals was obtained by total-body exposure to 400 roentgen of X-rays (250 KeV, 8 mA; filter: 1 mm Cu.)

RESULTS

Transplantability of cells propagated in vitro *or by serial passage* in vivo

The tissue culture strain MB VIA was originally highly tumorogenic if injected into syngeneic hosts but the tumor-producing capacity had decreased after 15 years of propagation *in vitro* [9]. At the start of these experiments the strain was aged 23 years and it did not give rise to tumors following inoculation of as many as 10^7 cells. However, tumors developed within 14 days following injection of 10^6 cells into the eye-chamber of untreated mice. Also, lethal tumors developed in animals irradiated with 400 r of X-rays and challenged with 10^6 cells by intraperitoneal or subcutaneous injection. Apparently, an immunological barrier has been developed between the cells in tissue culture and the parental inbred strains of the host animals.

However, the tumors that developed in immunosuppressed animals injected with tissue culture cells, were transplantable in untreated, syngeneic animals and could be serially passaged weekly. Moreover, the newly established strain of transplantable tumor cells appeared to be non-specific or "universal", for the cells were accepted by allogeneic mice as well.

Agglutination of cells propagated in vitro *or* in vivo

Agglutinability of MB VIA cells grown in tissue culture was high and comparable to the agglutinability of polyoma transformed hamster cells tested in a similar assay [12]. In Fig. 1, curve 1 the agglutination times are represented for cells in tissue culture assayed at various concentrations of Con A. However, growing these cells *in vivo* as transplantable tumors, strongly reduced their agglutinability. Fig. 1, curve 5, shows that cells isolated from tumors had approximately 10 times longer agglutination times at the various Con A concentrations tested than the same cells propagated *in vitro* (Fig. 1, curve 1). This difference was not due to the Isopaque-ficoll gradient centrifugation to which cells from tumors were subjected prior to the agglutination assay. A similar treatment of tissue culture cells did not reveal any effect on subsequent agglutination assay.

Cells propagated *in vivo* as subcutaneous or ascites tumors were treated with trypsin prior to the agglutination assay. Depending on the

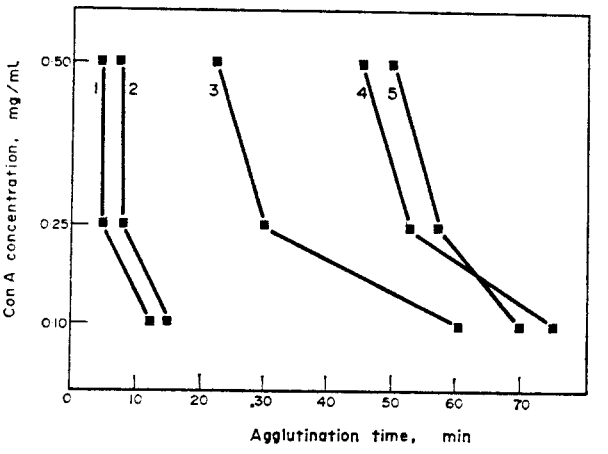

Fig. 1. Agglutination time of lymphosarcoma cells. Curve 1: cells permanently grown in tissue culture; curve 5: cells isolated from transplantable tumors; curve 2: cells isolated from tumors and propagated for 5 days in tissue culture; curve 3: idem for 3 days; curve 4: idem for 3 days but kept at 4°C. Duplicate determinations in 2 experiments.

enzyme concentration used, agglutination time was reduced by this treatment to the low level observed in the same cells propagated in tissue culture (Fig. 2). These results suggested that in cells propagated in tumors, the Con A agglutination sites had been made cryptic by a trypsin-sensitive layer. Therefore, it was tested whether these sites were covered by antibodies in the following assays:

(a) Smears of cells from tumors were incubated with conjugated anti mouse globulin as described under Material and Methods. No mouse immunoglobulins could be detected on the surface of tumor cells.

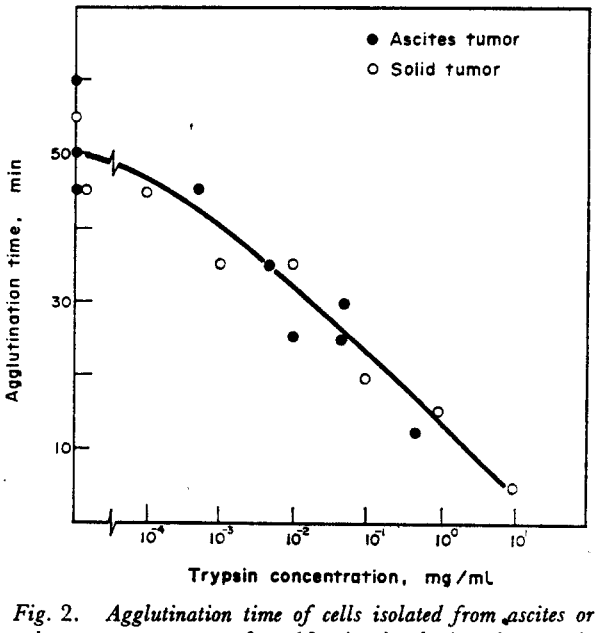

Fig. 2. Agglutination time of cells isolated from ascites or subcutaneous tumors after 10 min incubation in trypsin solutions of indicated concentration. Con A conc.: 0·5 mg/ml.

(b) Cells from tumors were incubated in glycine-HCl buffer at pH 2·8 to dissociate eventual antibodies from the cell surface. After washing of cells in HBSS at pH 7·2, cells were tested in the agglutination assay with Con A. No effect of pretreatment at low pH on agglutinability could be detected.

(c) Cells propagated *in vitro* with high agglutinability were incubated in serum or ascitic fluid from tumor-bearing animals to adsorb possible antibodies. After washing in HBSS, agglutination with Con A was measured. This treatment did not reduce the agglutinability of cells in tissue culture.

From these results it was concluded that the cryptic state of Con A agglutination sites of cells propagated in tumors was not due to masking of these sites by antibodies.

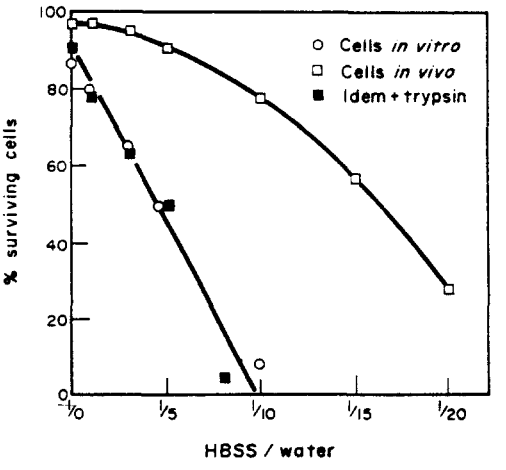

Fig. 3. The effect of hypotonic treatment on the viability of lymphosarcoma cells propagated in vitro *or* in vivo *in transplantable tumors. Trypsin treatment: 60 min at 0·2 mg trypsin/ml.*

The effect of hypotonic treatment

The assumed trypsin-sensitive layer on the surface of tumor cells propagated *in vivo* was studied by changing the surface as a consequence of hypotonic swelling. Preliminary experiments had indicated that maximal swelling was reached within 10 min of incubation in diluted buffer.

First, the effect of hypotonic treatment on cell viability was tested by incubation of cells in diluted HBSS for 10 min and subsequent resuspension in isotonic HBSS containing 0·5% trypan blue. As shown in Fig. 3, cells propagated *in vivo* were much more resistant to hypotonic killing than cells propagated in tissue culture. However, cells from tumors became as sensitive as tissue culture cells following treatment with trypsin.

Next, the effect of hypotonic treatment on agglutination with Con A was tested. Cells were isolated from tumors and suspended in diluted HBSS for 10 min. After this treatment, cells were resuspended in HBSS of the same dilution containing the agglutinin, and the agglutination time was measured. Also, samples of swollen cells were resuspended in isotonic HBSS to test for the reversibility of the effect. Microscopic inspection assured that swollen cells returned to their normal size if resuspended in isotonic HBSS.

The results in Table 1 show that cells grown *in vivo* became more agglutinable following hypotonic swelling. The effect was proportional to the degree of swelling and fully reversible if cells were returned to isotonic HBSS. The agglutination time of control cells was somewhat lower than routinely observed

Table 1. Agglutination time with Con A (0.5 mg/ml) of tumor cells suspended in or pretreated with diluted Hanks' buffered salt solution (HBSS)

Solution	Relative surface area	Agglutination time (min) in diluted HBSS	Agglutination time (min) resuspended in isotonic HBSS
HBSS (control)	1	I. 25–35*	—
		II. 30–35	
HBSS/water 1 : 1	1·6	I. 10	I. 30–35
		II. 10–15	II. 25–35
HBSS/water 1 : 9		I. <5	I. 25–35
	3·3	II. <5	II. 35

* I. Ascites tumor;
II. Subcutaneous tumor;

(*viz.* 25–35 min instead of 40–50 min). This discrepancy is ascribed to the repeat washings involved in this type of experiment.

Agglutination of cells from tumors explanted in tissue culture

Cells were isolated from subcutaneous or ascites tumors and seeded in Erlemeyer flasks in culture medium. As shown in Fig. 1 by the curves 2, 3 and 5, these cells gradually regained the high degree of agglutinability observed in the cells of strain MB VIA from which the tumors originated (Fig. 1, curve 1). The effect was prevented by storing the cells at 4°C (Fig. 1, curve 4). In experiments of this type, considerable variation was observed in a total of 25 tumor explantations. On the average, maximal agglutinability was obtained after 4 to 5 days of culture *in vitro* but extreme values of 1 and 10 days were observed as well.

To test the effect of cell density, cultures of different density were initiated from an explant of an ascites tumor. The cells then sedimented to the bottom of the culture flask but did not attach there. Also, cell suspensions were transferred to culture flasks (milk dilution type) mounted in a roller incubator to prevent sedimentation of cells. The results in Fig. 4 show that agglutinability increased more rapidly in dense cultures (Fig. 4, curves 2, 3 and 4) but did not do so if cells were kept in suspension (Fig. 4, curve 1). Therefore, we suggest that the return to high agglutinability depends on *local cell density* rather than on the ratio of cell number to medium volume.

Though the return to high agglutinability of explanted tumor cells was inhibited by storage at 4°C, a correlation between mitotic activity and unmasking of agglutination sites could not be established. Agitated cultures which returned much faster to a high degree of agglutinability did not proliferate better than standing cultures.

Loss of transplantability of explanted tumor cells

When cells from ascites or subcutaneous tumors were explanted in tissue culture, they

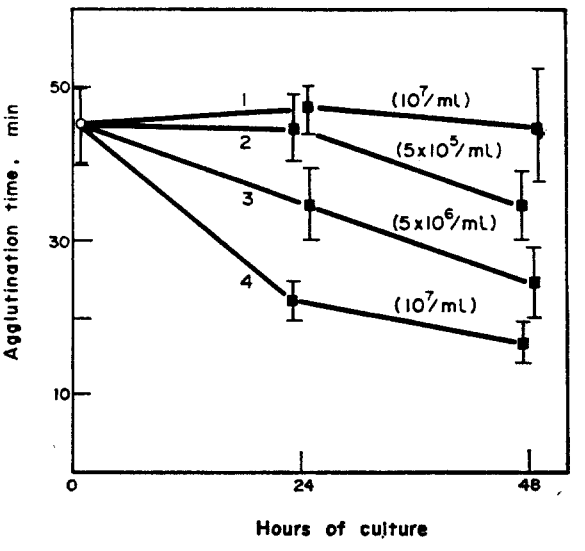

Fig. 4. The effect of cell density and culture conditions on the agglutinability with Con A of ascites cells explanted in tissue culture. Con A conc.: 0·5 mg/ml. Curve 1: agitated culture; curves 2, 3, and 4: standing cultures of cell density indicated between parentheses. Mean and s.d. from triplicate determinations in two experiments.

gradually lost the potential to develop lethal tumors upon reinoculation into normal mice. This loss of transplantability was correlated with the observed increase in agglutinability of tumor cells explanted *in vitro*. Cells seeded at high density (i.e. 5×10^6/ml) lost tumorogenicity in normal mice within a period of 3 days. On the other hand, cells grown at low density ($0{\cdot}5 \times 10^6$/ml) neither assumed full agglutinability nor lost transplantability after 7 days of *in vitro* culture as shown in Table 2.

To seek more support for the inversed correlation between agglutinability and transplantability, ascites tumor cells were explanted in culture flasks and grown under conditions which have differential effects on the reappearance of agglutinability such as shown in Fig. 4. The cells were subsequently injected into normal and 400-r irradiated mice to test transplantability. The results in Table 3 show that transplantability of explanted cells was better preserved under culture conditions which inhibited the return to short agglutination times such as storage at 4°C or in agitated

Table 2. The effect of tissue culture of explanted ascites tumor cells on agglutinability and transplantability

Days of culture *in vitro* ($0{\cdot}5 \times 10^6$ cells/ml)	Agglutination time in min with Con A: 0·5 mg/ml	0·25 mg/ml	Mean survival time (days ± s.d.)
0	65	65	11 ± 1
3	60	65	12 ± 1
7	25	35	23 ± 3
10	5–10	10–15	>60 (tumor free)

Table 3. The effect of culture conditions on transplantability of tumor cells explanted in tissue culture

Conditions of culture (5×10^6 cells/ml)	Agglutination time (min) with Con A (0·5 mg/ml)	Host animal	Mean survival time of hosts in days ± s.d. (inoculum: 10^6 cells)
A. Freshly isolated tumor cells	45	normal	11 ± 1
B. Idem		irradiated	10 ± 2
C. Cultured for 48 hr. at 37°C. No agitation	15	normal	>60 (tumor free)
D. Idem		irradiated	13 ± 1
E. Idem at 4°C	40	normal	12 ± 2
F. Idem at 37°C With agitation	35	normal	12 ± 1

cultures. However, cells that had lost transplantability in normal mice were still transplantable in irradiated animals similar to the MB VIA cells permanently grown *in vitro* (Table 3, D).

DISCUSSION

The experiments described in this report have indicated that MB VIA mouse lymphosarcoma cells, cultivated for many years in tissue culture, were highly agglutinable with Con A. Moreover, as these cells were rejected by normal mice but not so by irradiated mice or upon implantation in the eye-chamber, we propose that Con A agglutination sites as well as transplantation antigens have strong expression in these cells *in vitro*.

However, in transplantable subcutaneous or ascites tumors, developed from MB VIA cells by a single passage through an irradiated animal, Con A agglutination sites were masked and so were transplantation antigens since cells from tumors were transplantable in allogeneic as well as in syngeneic mice. Finally, when cells propagated in tumors were explanted into tissue culture, they reassumed agglutinability with a parallel loss of transplantability in normal mice (Tables 2 and 3).

These observations are readily explained by the assumption that tissue culture cells injected into an irradiated mouse were provided with a coat shielding Con A agglutination sites as well as transplantation antigens. This surface coat was gradually lost upon explantation into tissue culture. The existence of a surface coat which facilitates transplantability by reducing antigenic expression has been postulated for many other cells [6, 7, 8].

The actual existence of surface coating material on lymphosarcoma cells propagated as tumors was demonstrated by the unmasking of Con A agglutination sites following trypsin treatment (Fig. 2) and by the resistance to hypotonic disruption caused by trypsin-sensitive material (Fig. 3). The reversible exposition of Con A binding sites by hypotonic swelling (Table 1) suggests that the surface coat was made up of a dilatable network of macromolecules. This interpretation of the structure of the surface coat agrees with the negative outcome of the tests for covering antibodies. Moreover, it could explain why the surface coat did not impose growth control on the malignant cells in contrast with the effects of trypsin-split Con A molecules on virus-transformed cells [2]. Specific binding to agglutinin receptor sites rather than shielding of agglutination sites and antigens may be an essential feature of a coat with growth regulating properties [compare ref. 13]. Anyhow, our results confirm the conclusion reached by other authors [5] that the cryptic state of Con A agglutination sites is not an exclusive property of normal cells. When cells from tumors were explanted *in vitro*, an increase in agglutinability was observed (Fig. 1; Table 2). This return to high agglutinability was inhibited if cells were stored at low temperature (Fig. 1, curve 4) and depended also on local density (Fig. 4). The same conditions that inhibited the reappearance of high agglutinability of explanted tumor cells also prevented the loss of transplantability in normal mice (Table 3).

To explain these results, we propose that during *in vitro* culture the surface coat was gradually removed by proteolytic enzymes released by the tumor cells into their microenvironment. Culture of explanted tumor cells at low temperature would inhibit this process and agitation would prevent accumulation of released enzymes in the microenvironment of the cells. Consequently, these conditions would inhibit the breakdown of the surface coat and thus the exposition on Con A agglutination sites and transplantation antigens (Fig. 4; Table 3). The increased release of catabolic enzymes by neoplastic cells both *in vitro* [14] and *in vivo* [15] has been reported. This conclusion has been supported by the findings of Belpomme *et al.* [16] who observed a marked increase in antigenicity of human lymphoblastic leukaemia cells during *in vitro* culture.

As yet it is not known whether the assumed surface coat arose from selection of "coated" cells in the primary inoculum, from *de novo* synthesis by the injected cells or whether the composing molecules were withdrawn from the body fluids of the host animal as suggested by Apffel and Peters [6]. To solve this question, the agglutination assay may serve as a useful tool for a rapid detection of a surface coat prior to transplantation experiments.

In summary, our results have confirmed the involvement of a surface coat in the establishment of (non-specific) transplantability of tumor cells. Moreover, agglutination with Con A has been shown to be a tool of possible value to further analyse the origin and structure of such a coat.

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Ultrastructural, Cytochemical and Biochemical Changes Occurring During Syngeneic Macrophage–Lymphoma Interaction *in vitro*

F. LEJEUNE* and R. EVANS†

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London S.W.3, England

Abstract—*Immune peritoneal macrophages (IPM) obtained from DBA/2 mice hyperimmunized against syngeneic L5178Y lymphoma differed from normal macrophages (NPM) in that they were larger, they exhibited more small lysosomes in the Golgi region and they had a higher intracellular acid phosphatase activity. When cultures of IPM and NPM were mixed with the lymphoma cells at a ratio of 40:1, there was an increase in the number of small lysosomes reaching a peak level at 2 hr. Although this occurred in both macrophage types the actual increase was greater in IPM and was followed by lysosomal fusion. There was no detectable biochemical increase of acid phosphatase in either IPM or NPM before 6 hr.*

At a 1:1 ratio, adhesion of L5178Y cells was rapid and strong, although there was no specific ultrastructural damage to the tumour cells before 24 hr.

The data are discussed in relation to the immunological specificity of target cell growth inhibition by IPM and the changes taking place in the macrophages.

INTRODUCTION

THE INVOLVEMENT of macrophages in allograft rejection has been reported by several investigators *in vivo* and *in vitro* [1, 2]. *In vitro* studies have demonstrated that macrophages from allografted mice were cytotoxic towards the target cells [1–5], and electron microscopy studies have revealed intimate cell-to-cell contacts during allograft rejection *in vivo* [6] and during *in vitro* cytotoxic reactions [7].

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*Present address: Institut Jules Bordet, 1 rue Héger-Bordet, 1000 Brussels, Belgium.

†Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Belmont, Sutton, Surrey, England.

Until recently there was no direct evidence that macrophages were involved in syngeneic tumour rejection but the evidence now suggests that they may play a vital role in the effector side of rejection. Evans and Alexander [8] reported that peritoneal macrophages from mice immunized with the irradiated syngeneic lymphoma L5178Y [9] inhibited growth of these lymphoma cells in an immunologically specific manner, a reaction dependent on cell-to-cell contact.

In this report, the ultrastructural, biochemical and cytochemical changes following contact between macrophages from normal and immunized mice and the specific syngeneic L5178Y target lymphoma cells were studied, with a view to gaining information on the possible mechanism(s) involved during the process of growth inhibition.

MATERIAL AND METHODS

Animals

Mice of the DBA/2 inbred strain, 8 to 10 weeks old were used as a source of peritoneal macrophages and for routine growth of the syngeneic L5178Y lymphoma, which grows as an ascites tumour following intraperitoneal implantation. Pure-line CBA mice were used to grow the syngeneic TLX5 lymphoma, also in the ascitic form.

Cultures

The preparation of peritoneal macrophage cultures has been fully described elsewhere [8].

Macrophages from normal control mice will be referred to as normal peritoneal macrophages (NPM) and those from immunized mice as immune peritoneal macrophages (IPM).

The L5178Y and TLX5 cells were harvested from mice, washed three times in Fischer's medium and resuspended at a concentration of 10^5 cells per ml of growth medium. Both grew in suspension, the L5178Y with a mean generation time of approximately 13·0 hr, the TLX5 lymphoma with one of 16 hr.

Immunization

DBA/2 mice were given three i.p. injections of 10^7 irradiated L5178Y cells, as described previously [8], and macrophages were harvested and cultured 10 days after the final injection.

Electron microscopy

Monolayers of macrophages were fixed *in situ* with 1% freshly distilled glutaraldehyde in 0·08M cacodylate buffer at pH 7·2 for 30 min at room temperature; kept overnight at 4°C with two changes of buffer, and then postfixed with 1% osmium tetroxide in veronal acetate buffer for 60 min at 4°C. After a brief wash in buffer the monolayer was dehydrated in ethyl alcohol and the plastic dishes filled with two changes of Epon 812 (50%A, 50%B, [10]). After polymerization for 24 hr at 60°C, randomly selected areas of the embedded dishes were cut out and cut parallel to the surface. The sections were stained in an alkaline lead solution [11] and photographed on a Philips EM300 microscope. For each estimate, lysosomes in the Golgi region of 10 to 20 macrophages were counted. The cytoplasmic area measured was at least 200 μ^2, and the lysosome counts were expressed as the number/10 μ^2 of cytoplasm.

Cytochemical methods for acid phosphatase

1. *Light microscope method.* The macrophage monolayers were fixed in 1% formaldehyde in 0·1M cacodylate buffer with 0·2% Ca Cl2 for 30 min at 4°C. Naphtol AS–B1 phosphate was used as a substrate [12]. Quantitative determination of the enzyme activity was made by measuring the light absorbance at 500 mμ in 100 individual cells using a microspectrophotometer.

2. *Electron microscopical method.* The macrophages were fixed in 1% glutaraldehyde, as above, but at 4°C for 30 min. The dishes were incubated in prewarmed Gomori substrate [13] after a treatment with 0·88 M sucrose [14] for 30 min at 4°C before incubation.

Biochemical method for acid phosphate

One millilitre of 1% Triton X-100 in 0·15M veronal acetate buffer at pH 5·0 was poured onto each monolayer and the dish stored in the deep-freeze. After thawing, 0·3 ml from duplicate cultures were incubated with 1·5 ml of the same substrate as used for cytochemistry (see above) for 2 hr at 37°C. The reaction was estimated by extraction of the dye [15] with ethyl acetate and read spectrophotometrically at 500 mμ.

β-glucuronidase biochemistry

The only alteration to the method for determination of acid phosphatase activity was that the substrate was made up with naphthol AS–LC–βD glucuronide and hexazonium pararosanilin [16].

RESULTS

1. *Electron microscopy*

The *in situ* embedding revealed an ultrastructural difference between NPM and IPM cultures before and during the syngeneic tumour challenge which are listed in Table 1 and may be summarized as follows:

(a) *Macrophages cultured for 24 hr before challenge.* Table 1 summarizes data from sections cut through the Golgi region and demonstrates that IPM were larger, exhibited a higher frequency in changes of polarity where the nucleus and Golgi region appeared to move in opposite directions, and a higher number of lysosomes compared with NPM (Figs. 1 and 2).

(b) *Macrophages challenged with lymphoma cells at a ratio of* 40 : 1. IPM have been shown to inhibit growth of lymphoma cells at this ratio [8], and although it was apparent that the growth inhibitory mechanism had been initiated before 24 hr there was no ultrastructural difference between the lymphoma cells pelleted

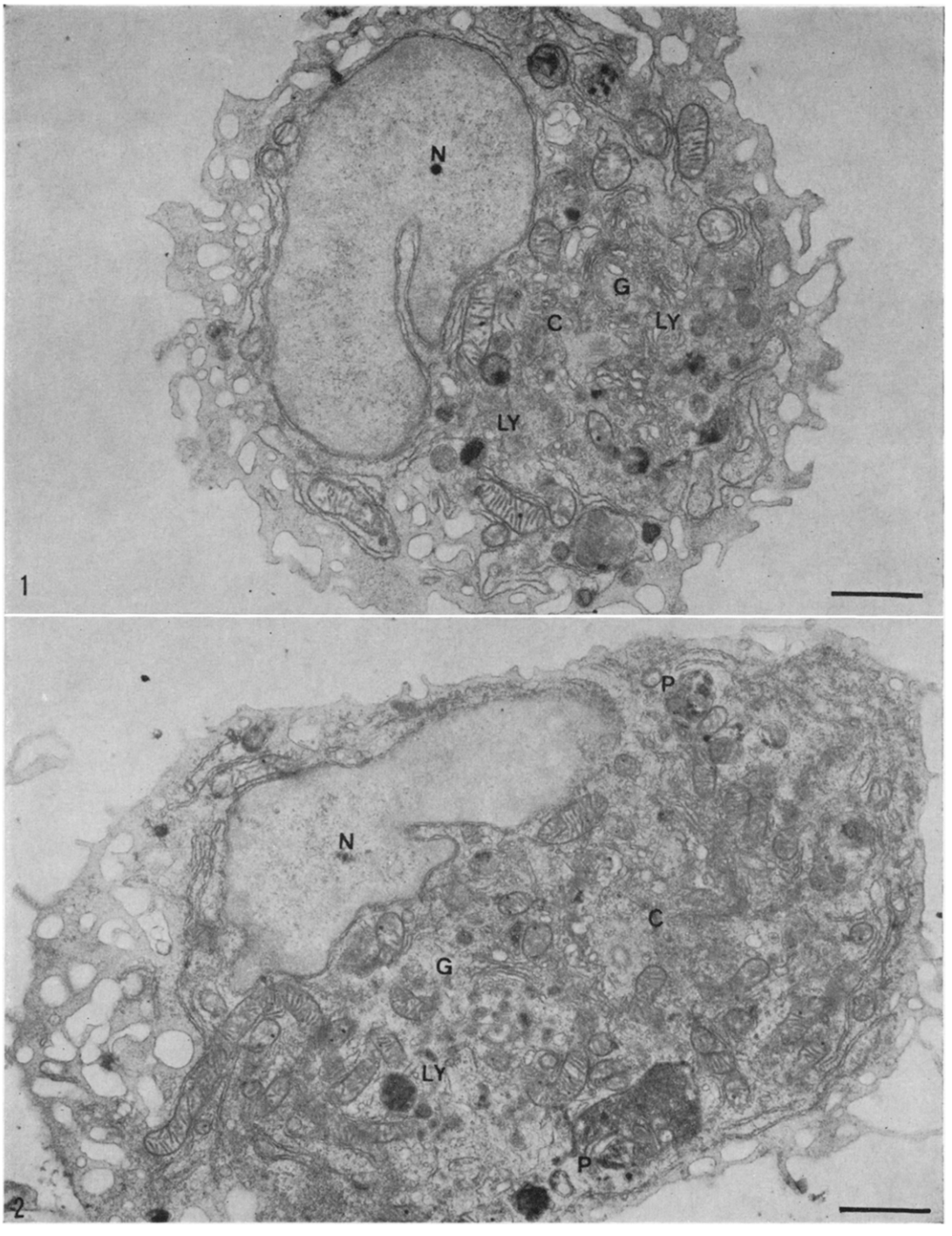

Fig. 1. *Normal DBA/2 peritoneal macrophage (NPM) cultured alone for* 24 *hr showing the typical microvilli. Compared with Fig.* 2, *this cell is unusually polarised with an indented nucleus (N), centrosome (C), Golgi region (G) and some lysosomes (LY).* × 17·000. *Bars represent* 1 μ.

Fig. 2. *Immune peritoneal macrophages (IPM) cultured for* 24 *hr. This cell is different from NPM (Fig.* 1) *in that it is larger and exhibits more small lysosomes (LY) round the centrosome (C) and the Golgi region (G). There are* 2 *phagocytic vacuoles (P).* × 17·000.

(to face p. 550)

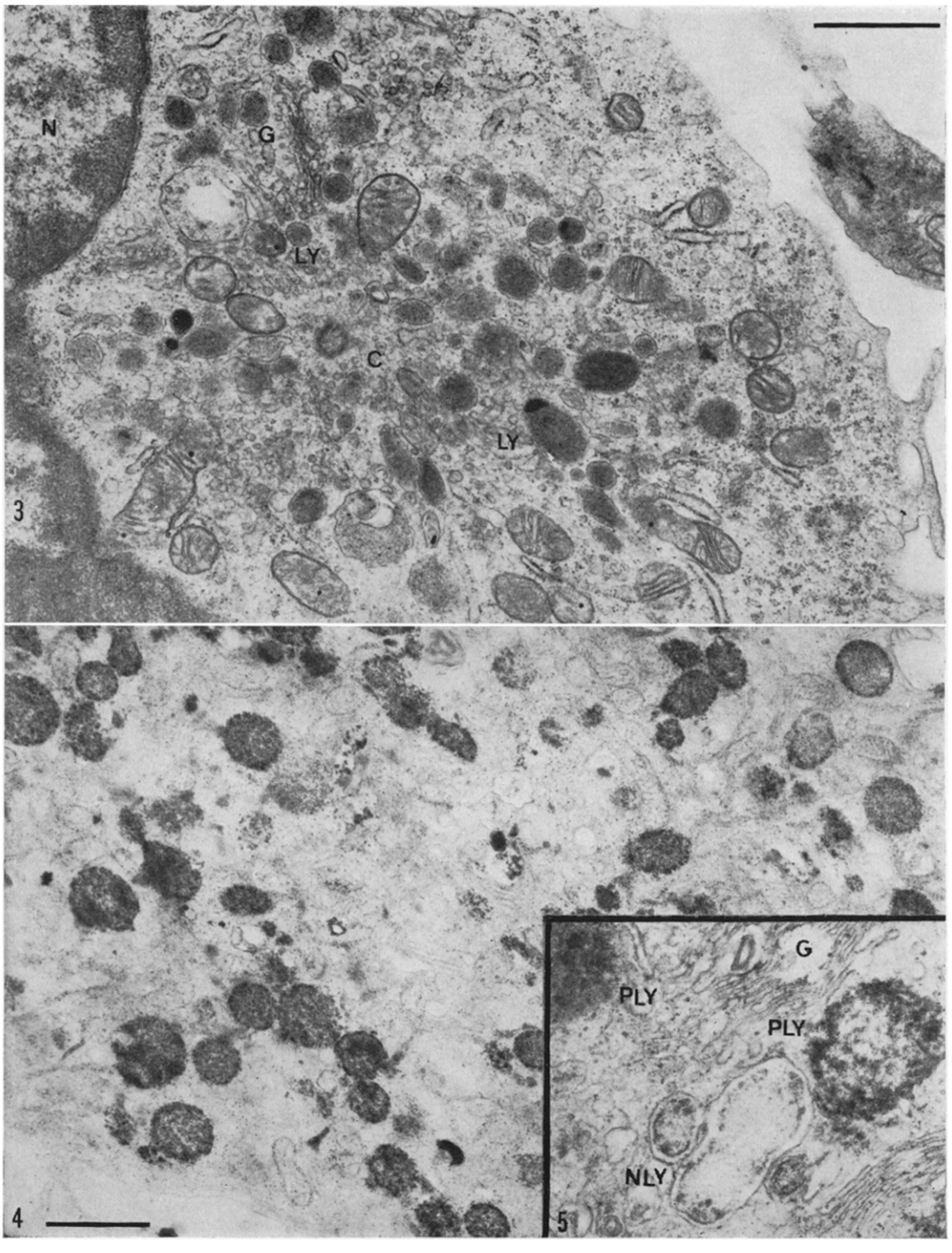

Fig. 3. *IPM cultured for* 2 *hr with L*5178*Y lymphoma at* 40:1 *ratio. Compared with unstimulated IPM* (*Fig.* 2) *there is an increase of small lysosomes* (*LY*) *forming a rosette around the centrosome* (*C*). *N: nucleus; G: Golgi region.* × 24·000.

Fig. 4. *IPM cultured under the same conditions as that in Fig.* 3 *but reacted for acid phosphatase. In this section all lysosomes* (*LY*) *are strongly positive.* × 20·000.

Fig. 5. *Identical condition as Fig.* 4 *but at a higher magnification* (× 60·000) *showing* 2 *lysosomes negative for acid phosphatase* (*NLY*) *and* 2 *positive lysosomes* (*PLY*). *G: Golgi region.*

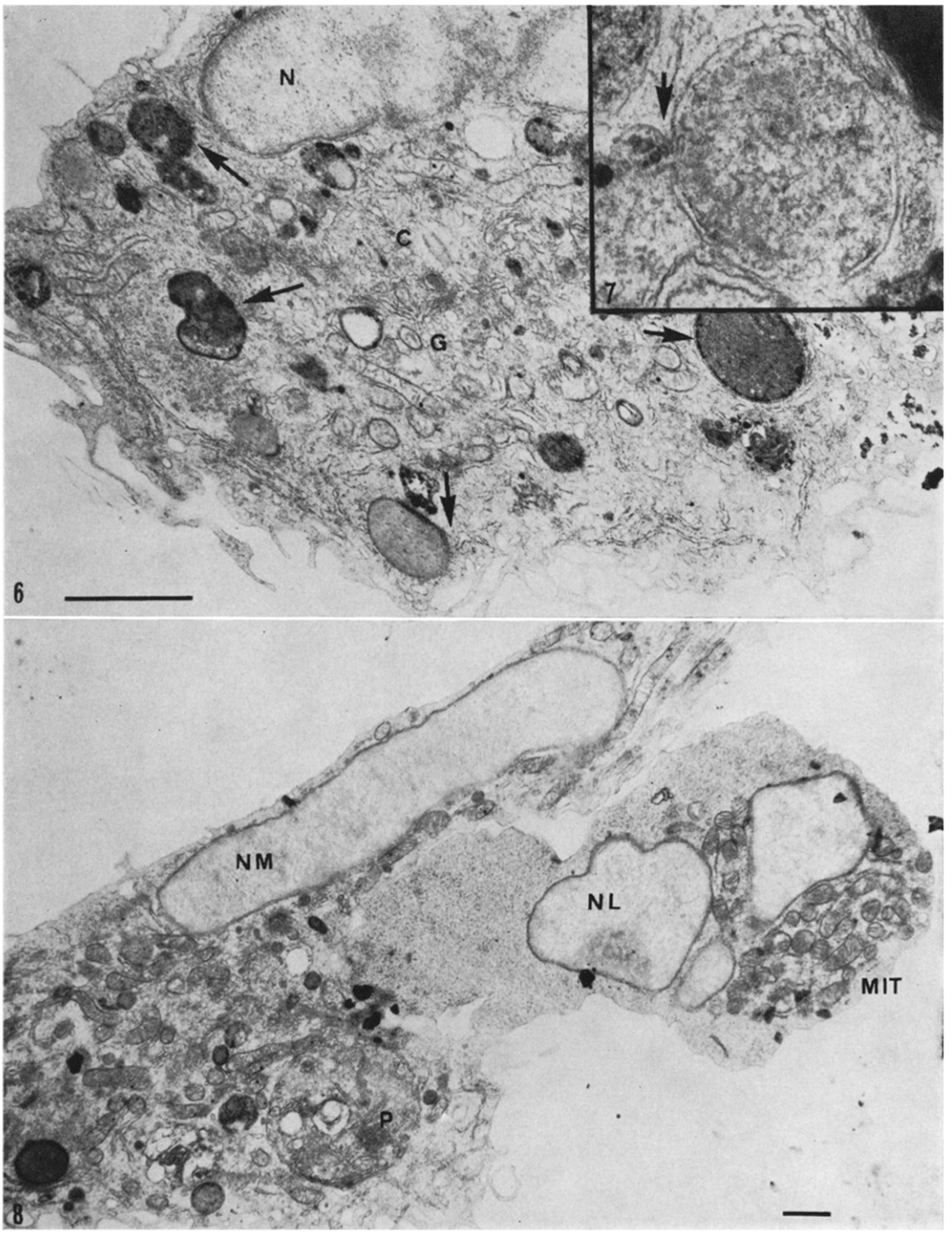

Fig. 6. *IPM cultured with L5178Y lymphoma for* 6 hr *at* 40:1 *ratio. There are a very few small lysosomes round the centrosome* (*C*) *and the Golgi region* (*G*), *but larger lysosomes are present at the cell periphery* (*Arrows*). × 24·000.

Fig. 7. *Same conditions as Fig.* 6. *A small lysosome appears to be fusing* (*arrow*) *with a larger lysosome.* × 60·000.

Fig. 8. *IPM cultured with L5178Y lymphoma at* 1:1 *ratio. A single IPM is in contact with the lymphoma cell, of which the cytoplasmic area surrounded by the macrophage is free of organelles, except polyribosomes. All the mitochondria* (*MIT*) *are packed opposite to the contact area. P = phagocytic vesicle.* × 9·600.

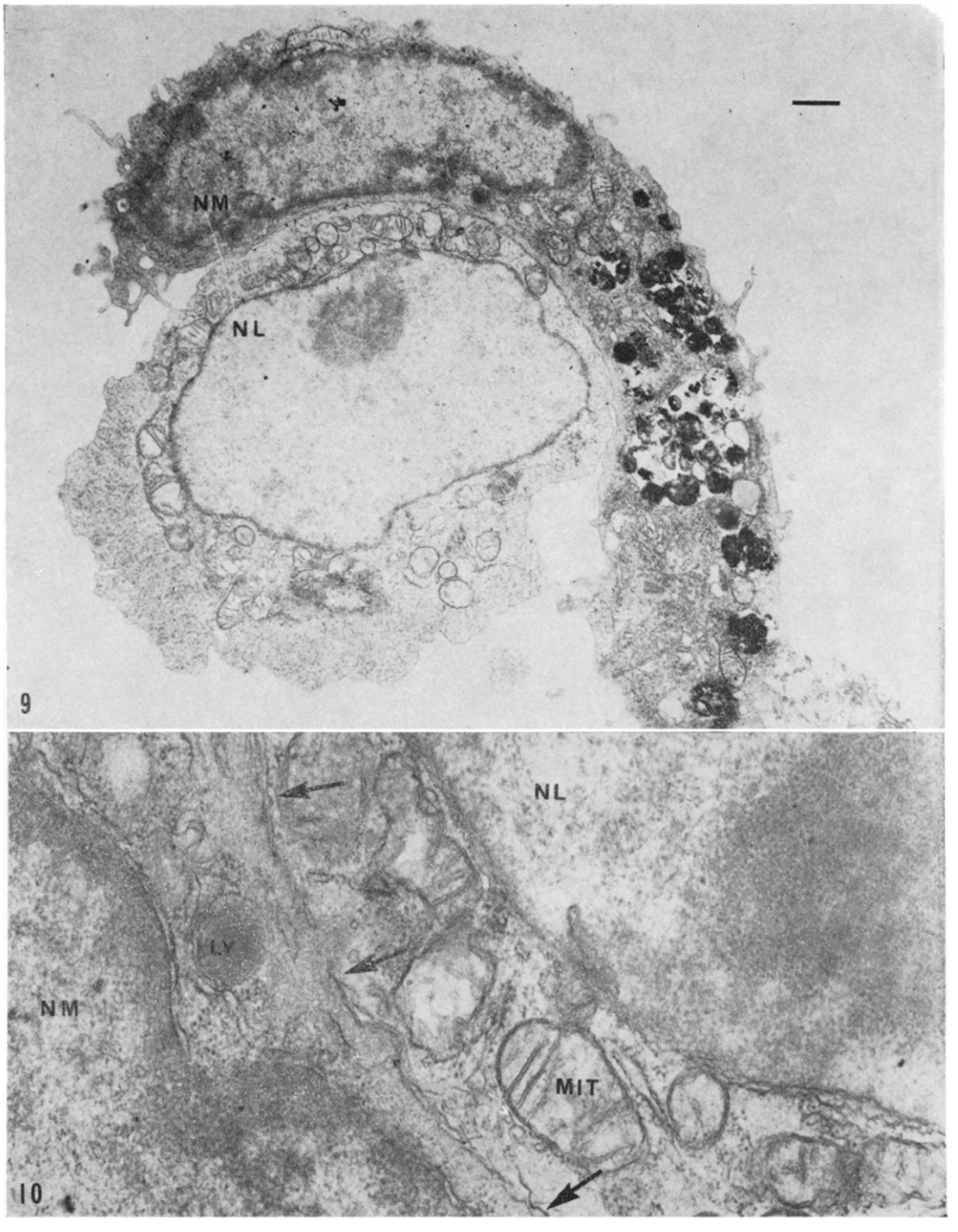

Fig. 9. *Same conditions as Fig.* 8. *Half of the lymphoma cell circumference is surrounded by and in very close contact with the IPM.* × 9·600.

Fig. 10. *Higher magnification of the Fig.* 9 *contact area showing a close apposition of the membranes* (*arrows*) *and disappearance of cell organelles in the macrophage along the contact area.* × 45·000.
NM = IPM nucleus; *MIT = Mitochondria*; *NL = Lymphoma nucleus*; *LY = Lysosome.*

Table 1. Comparison of normal peritoneal macrophages (NPM) and immune peritoneal macrophages (IPM) before and after adding L5178Y lymphoma cells

	Time (hr)	Size	Polarity of nucleus %	Lysosomes in Golgi Number per $10\mu^2$	Permanent contacts between L5178Y and macrophages after washing monolayers	Macrophages showing engulfment of lymphoma debris (%)
NPM	0*	$9\mu \pm 3$	20	5 (4–6)		15
IPM		$14\mu \pm 4$	80	15 (2–25)		10–20
NPM + L5178Y						
40:1	2		70	12 (3–21)	0	25
	6		70			25
NPM + L5178Y						
1:1	2		90		0	35
	6		90		+	60
IPM + L5178Y						
40:1	2		90	32 (13–60)	0	15
	6		90			15
IPM + L5178Y						
1:1	2		90		+	75
	6		90		+	70

*0 hr: macrophage cultures prepared 24 hr previously

Table 2. Intracellular acid phosphatase in different experiments (Number mμM phosphate/6×10^5 PM/Hr)

	Experiment No: 1	2	3	4
IPM (against L5178Y)	50	52	100	58
NPM	25	27	15	18
Intracellular β-glucuronidase				
	Experiment No: 4			
IPM	160			
NPM	35			

from IPM and NPM recovered up to this time. Consequently, we decided to study changes occurring in the macrophages within this period of incubation. At this ratio of 40 : 1, there was a demonstrable change in ultrastructure in both IPM and NPM within 2 hr of challenge. It consisted of a two-fold increase of small lysosomes (0·15–0·3 μ) in the Golgi region of the macrophages (Figs. 3 and 4). The number of small lysosomes seen after various times is plotted in Fig. 11; the number reached a maximal level by 2 hr but thereafter decreased. In IPM only, the decrease over 22 hr was accompanied by the formation of large lysosomes (> 0·5 μ) at the cell periphery (Fig. 6); this was apparently due to the fusion of small lysosomes (0·15–0·30 μ) (Fig. 7). The formation of the large lysosomes by fusion did not occur in NPM. Fig. 12 illustrates that during the initial 2 hr after challenge the number of lysosomes per 10 μ^2 in IPM increased dramatically compared with IPM not challenged with L5178Y cells. Very few lymphoma cells remained firmly adherent to either IPM or NPM at this ratio of challenge after washing the cultures.

(c) *Macrophages cultured with lymphoma cells at a ratio of* 1 : 1. This ratio did not allow cultivation of mixed cultures for longer than 6 hr because of nutrient problems, and under such cultural conditions much cell debris was seen. When the lymphoma cells were washed

off the macrophage monolayers by pipetting the medium over the macrophages, the remaining permanent contacts were examined. At 2 hr there were few contacts in IPM and none in NPM cultures. By 6 hr numerous contacts remained after washing IPM cultures; the contact consisted of a simple close apposition of the two cell membranes (Figs. 8–10). In most cases, the macrophage had partly surrounded the lymphoma cell, but there were neither interdigitations nor changes in the lymphoma membrane. The IPM showed sometimes a disappearance of cell organelles in the area close to the contact (Fig. 8). The lymphoma cells usually showed a packing of mitochondria in the region of cytoplasm directly opposite the contact (Fig. 8). The NPM cultures at this time also showed occasional contacts of a similar type seen in IPM. These events are also summarized in Table 1. IPM showed a quicker and stronger phagocytosis of the debris produced at this 1 : 1 ratio (Fig. 13) than NPM. The difference was most obvious at 2 hr, but not significantly different at 6 hr. The lysosomes in IPM were mainly large ($> 1\mu$) by 2 hr compared with the smaller type seen in IPM cultures at the 40 : 1 ratio (Fig. 8).

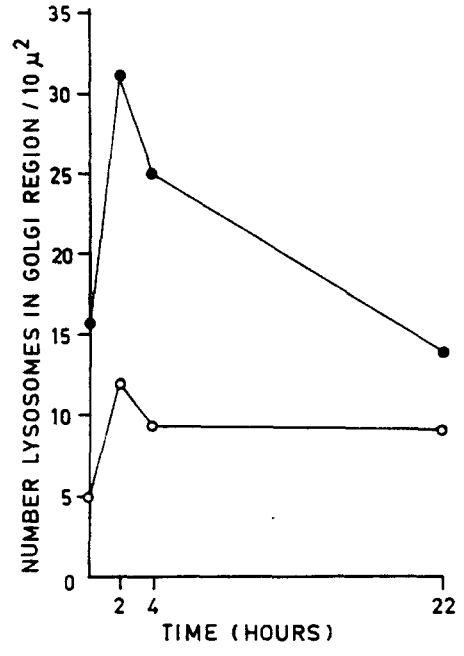

Fig. 11. Changes in lysosomes number in the Golgi region of macrophages during the mixed culture with L5178Y lymphoma cells at 40:1 ratio.
●—● *IPM* ○—○ *NPM*

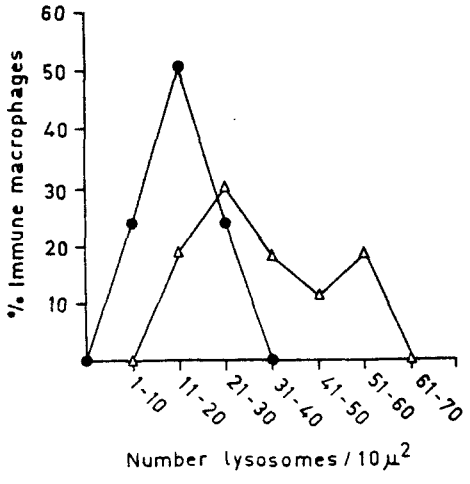

Fig. 12. Distribution of lysosomes number in IPM: ●—● *IPM cultured alone, 2 hr after changing the medium* △—△ *IPM cultured with L5178Y lymphoma for 2 hr at 40:1 ratio.*

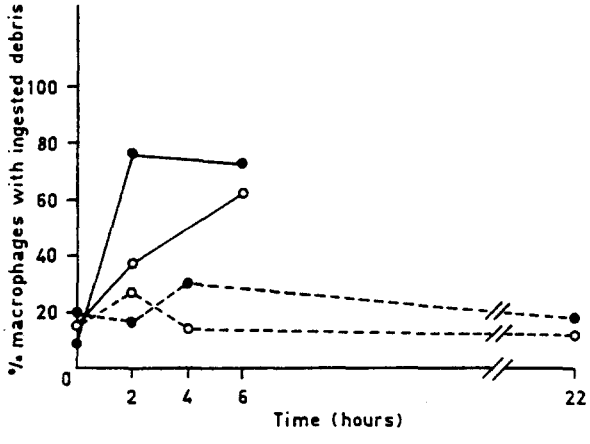

Fig. 13. Phagocytosis of lymphoma cells debris by peritoneal macrophages at two different ratios of macrophages/ lymphoma.
●—● *IPM* 1:1 ●--● *IPM* 40:1
○—○ *NPM* 1:1 ○--○ *NPM* 40:1

Cytochemical results

Electron microscopy. A strongly positive reaction developed in most of the lysosomes, both large and small, which were heavily stained (Fig. 4). Although occasional smooth reticulum cisternae were positive a few small lysosomes of the same diameter as the positive ones remained unstained (Fig. 5).

At a 40 : 1 ratio the total number of stained small bodies increased within 2 hr after challenge in both NPM and IPM, corresponding to the ultrastructural changes described earlier. Moreover, the increase shown after challenge with L5178Y cells and NPM was similar when cultures were challenged with the allogeneic CBA lymphoma TLX5, which was not inhibited by these IPM L5178Y specific [17].

Light microscope results: microspectrophotometry. Whereas NPM showed a normal distribution (Fig. 14), IPM showed a curve with two peaks; one of the peaks corresponded with that seen in NPM cultures while the other was of cells with an activity about twice as high.

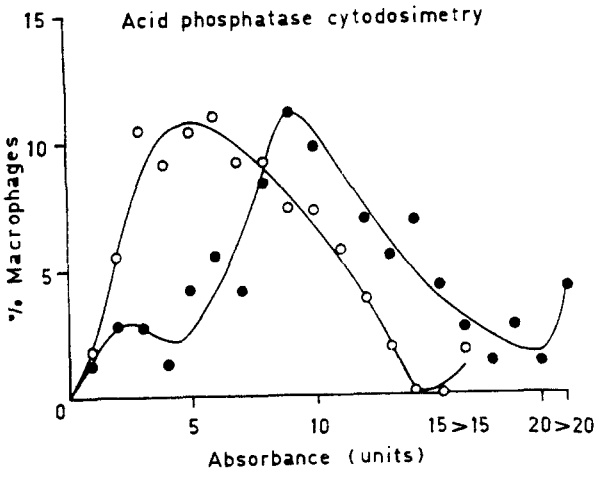

Fig. 14. *Acid phosphatase cytochemistry. IPM* ●—● *and NPM* ○—○ *monolayers are compared by microspectrophotometry.*

Biochemical results

Differences between NPM and IPM. The level of acid phosphatase in Triton-X extracts from macrophages (i.e. intracellular activity) which had been cultured in 10% serum for 24 hr (i.e. immediately before challenge with lymphoma cells) were at least twice as high in IPM compared with NPM (Table 2 and Fig. 15). The β-glucuronidase activity was measured in one experiment only and found to be four times greater in IPM. After challenge of macrophage monolayers at a ratio of 40 : 1 there was an increase in acid phosphatase activity in both IPM and NPM (Fig. 15). Moreover, the levels increased in parallel up to 22 hr; by 15 hr the level

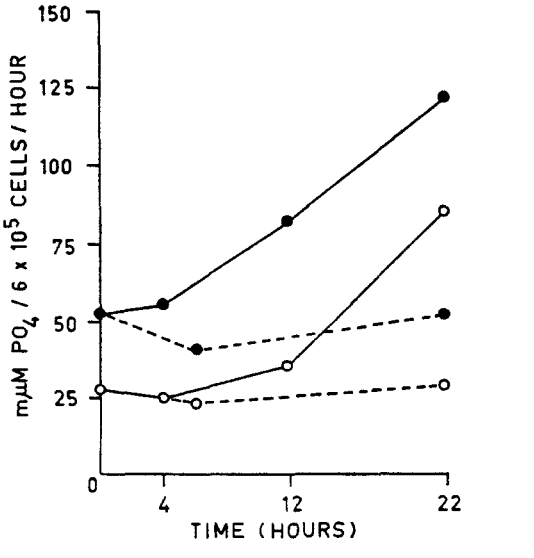

Fig. 15. *Acid phosphatase biochemistry. The macrophage intracellular acid phosphatase is measured at different culture times:* ●—● *IPM cultured with L5178Y lymphoma at* 40:1 *ratio.* ●--● *IPM cultured alone:* ○—○ *NPM cultured with L5178Y lymphoma at* 40:1 ○--○ *NPM cultured alone.*

in NPM reached that seen in IPM at zero time. When 1 : 1 ratios were used, the curves of activity were closely similar to the ones at a 40 : 1 ratio despite the 40 times stronger challenge. There was again no difference in the slope of the curves of IPM and NPM.

Measurements on the lymphoma pellet. The non-adherent cells in the mixed cultures were also assayed for acid phosphatase. The activity of these lymphoma cells remained constant through the whole incubation time of 24 hr at the 40 : 1 ratio; the pellets of the same size as used for assaying the macrophage activity had an activity of 5 ± 1 mμ MPO_4/hr.

DISCUSSION

The purpose of this report was to ascertain whether growth inhibition of L5178Y cells by immune macrophages from the peritoneal cavity of DBA/2 mice hyperimmunized with irradiated L5178Y cells resulted in ultrastructural, biochemical or cytochemical changes in either the macrophages or the target lymphoma cells.

Distinctive changes were seen in the macrophages but none was detectable in the target cells over the period of study. Consequently, the main interest of this study focused on differences arising in immune macrophages compared with non-immune.

Having established that immune and non-immune macrophages showed characteristic differences before monolayers were challenged with target lymphoma cells in that IPM (1) were larger than NPM, (2) the number of lysosomes in the Golgi region and their associated acid phosphatase activity, was almost twice as high as in NPM the changes occurring after challenge were viewed in terms of specific or non-specific reaction. Overall, the changes taking place in the immune macrophages were paralleled by those seen in non-immune macrophages, whether or not the target cells were the same as those used to immunize the host. Within 2 hr there was a dramatic increase in the number of small lysosomes, which was emphasized in immune macrophages, because of the higher starting levels. There was no increase in total acid phosphatase activity before 6 hr, although there was an increase in acid phosphatase-positive small lysosomes by 2 hr.

This parallelism indicates that the sequence of events seen was related only to the prevalent cultural conditions at particular times (such as

before and after the addition of lymphoma cells to the macrophage monolayers) and totally non-specific. That immune macrophages were biologically more active than NPM is of interest in that this could indicate that immune macrophages are better adapted for phagocytosis and for digestion of dying cells or debris, particulary during tumour rejection *in vivo*.

Notwithstanding this apparent lack of detectable specific lysosomal response by immune macrophages, the findings (1) that lysosomal increase was very rapid and (2) that there was a lapse of 4 hr between this increase and the rise of acid phosphatase activity are new, as far as we are aware. Others [26, 27] have reported similar overall changes during endocytosis, which would seem to be the main stimulus for increase in lysosomal numbers [14, 18–21], and also for the subsequent increased enzyme activity [22–25].

Whether there are enzymic systems directly involved either in the antigenic recognition by immune macrophages or during the process of growth inhibition remains to be explored. It seems unlikely that the activity of some enzymes is not specifically activated or depressed, and a study of such enzymes as membrane phospholipases or the cyclic AMP-associated adenyl cyclase might provide information.

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Absence of Fluorescent Y Bodies in Some Malignant Epithelial Tumours in Human Males: Preliminary Results

M. SELLYEI and L. VASS

Department of Pathology, Róbert Károly krt. Hospital, Budapest, XIII, Hungary

THE BRIGHT fluorescence on the long arm of the Y chromosome in human mitoses and interphase nuclei stained with quinacrine mustard [1, 2, 3] or quinacrine [4] seems to be an excellent tool for the detection of this chromosome, even in cases when its presence may not be proved by other morphological criteria [5]. The wide range of chromosomal rearrangements and the difficulties to obtain properly spread mitoses in most solid tumours interfere with their exact karyotyping. However, the quinacrine technique enables us to determine readily at least the fluorescent part of the male sex chromosome in human neoplasias [6].

We have investigated the occurrence of Y body (YB) in interphase nuclei of human malignant epithelial tumours from 18 males (Table 1) and 9 females. The material was obtained either by autopsy performed within 24 hr of death or surgically. In most cases the non-tumorous liver tissue or gastrointestinal mucosa were also investigated. A 0·5 cm^3 volume of tissues was minced by scissors and then thoroughly squashed. The squashed tissue, diluted in physiological saline, was filtered through gauze to obtain a homogeneous cell suspension. After centrifugation the pellet was fixed in methanol. Cell smears were stained with quinacrine hydrochloride according to Pearson *et al.* [4]. The coded slides were evaluated under a fluorescent microscope by two investigators A (M.S.) and B (L.V.) independently. Both investigators counted a minimum of 100 nuclei in each smear. The prevalence of tumour cells in smears was controlled by subsequent staining with haematoxylin. The types of tumours were determined by histological examination.

Both tumorous and non-tumorous cells of female origin had a frequency of nuclei with YB-like fluorescent spots lower than 10% ranging from 0 to 9. The percentage of YB containing nuclei from non-tumorous tissues of the males ranged from 19 to 71. Out of the 18 male tumour specimens there were 6 in which less than 10% of nuclei contained YB (Table 1). These cases we regarded as YB negatives. A further male tumour (case 10, Table 1) had an intermediate value of 8 and 13%

The chromatin in tumour cells generally formed coarser clumps producing a more intense fluorescence than those of the nuclei of normal cells (Fig. 1). Nevertheless, the YB, when present could be recognized easily in male tumours due to its intense fluorescence and shape. The small dark chromatin clump in haematoxylin stained nuclei, corresponding to the fluorescent YB, cannot be distinguished from numerous similar clumps either by its arm or by the grade of heteropycnosis (Fig. 2).

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Table 1. *Occurrence of YB in tumorous and non-tumorous cells of human males*

Case	Age (years)	Site of tumour	Percentage of nuclei with YB			
			Tumour cells		Non-tumorous cells	
			A	B	A	B
1	73	lung	12 (3)	12	ND	
2	74	lung	3	9	ND	
3	60	lymph node, metastasis from the lung	4	5	ND	
4	67	brain, metastasis from lung	46	34	41 (3)	31
5	83	lung	5	3	25	40 (4)
6	48	stomach	4	5	40	31
7	69	duodenum	4 (1)	2 (1)	58 (2)	28
8	75	gall bladder	84 (2)	34 (7)	19	19 (4)
9	50	rectum	23	31 (2)	39	49
10	58	liver	8	13	71	34
11	53	liver	69 (2)	69 (3)	54	40
12	64	liver	77 (13)	40 (5)	67 (11)	66 (9)
13	56	liver	33	35 (2)	71	44 (4)
14	68	liver	7	5	38	21 (2)
15	72	urinary bladder	42 (3)	58 (4)	ND	
16	79	vertebra, metastasis from prostate	24 (2)	31 (7)	ND	
17	38	left inguinal region	32	23 (5)	ND	
18	63	brain	46 (3)	24	21	37 (2)

Tumours of male hosts are YB negative in cases 2, 3, 5, 6, 7 and 14. A and B: values according to two investigators. In parentheses: percentage of nuclei with more than one YB. ND: not done. Lung tumours were anaplastic carcinomas in cases 1 and 2, squamous cell carcinomas in cases 3 to 5. Adenocarcinoma was found in cases 6 to 9 and 16. Liver tumours corresponded to hepatocellular carcinoma (cases 10 to 14). Transitional cell carcinoma was found in case 15, embryonal carcinoma of uncertain origin in case 17, malignant astrocytoma in case 18.

We assume that the loss of fluorescent YB in male human tumours takes place during malignant transformation. The disappearance of YB may be due either to Y chromosome loss or to deletion of the fluorescent segment of the Y chromosome. In fact, out of 6 cases of male Burkitt's lymphoma cultivated *in vitro*, Manolov *et al.* [7] found a single case without fluorescent Y chromosome. The present data obtained on interphase nuclei facilitate the selection of YB negative tumours for a further detailed analysis of metaphases. The latter is necessary to elucidate the real chromosomal basis of the absence of fluorescent YB in some male malignancies.

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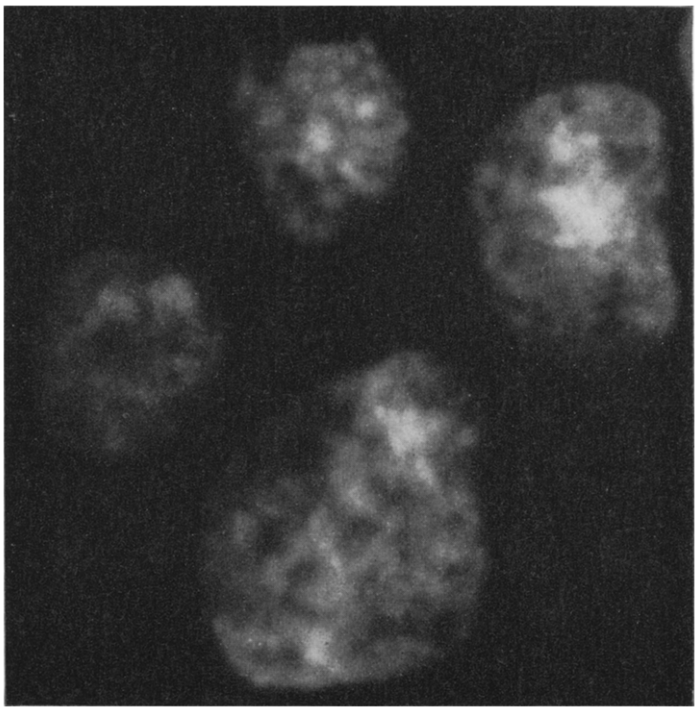

Fig. 1. *Group of YB negative malignant cells from a male host* (*case* 5, *Table* 1). *Note the uneven distribution of quinacrine binding chromatin clumps in the nuclei.*

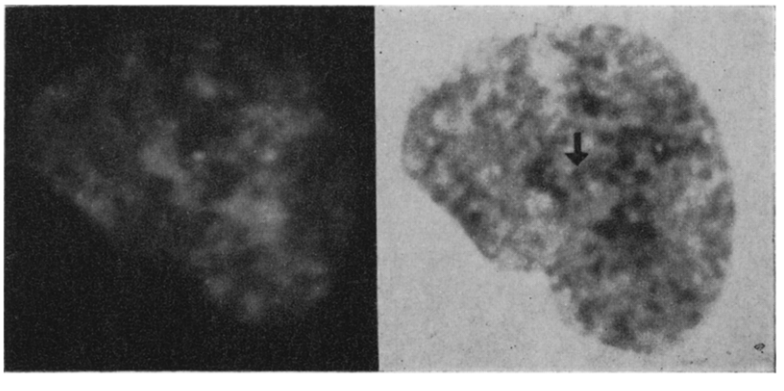

Fig. 2. *There are two YBs observed by quinacrine technique* (*left*) *in a tumour cell nucleus from the case* 12 (*Table* 1). *After haematoxylin staining* (*right*) *the chromatin is condensed in the corresponding areas* (*arrow*), *but without any peculiar features.*

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The Effects of Hyperthermia (42°C) on the Biochemistry and Growth of a Malignant Cell Line

JOHN A. DICKSON and DHANSUKH M. SHAH

Cancer Research Unit, Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle upon Tyne, England.

Abstract—*An* in vitro–in vivo *system has been used to study the effects of elevated temperature on the biochemistry and growth potential of a malignant cell line derived from a chemically-induced rat breast adenocarcinoma.*

Monolayer cultures of the cells in the lag phase of growth, and in the exponential phase of growth, were heated at 42°C *for up to* 9 *hr, and then returned to* 37°C *for study. Cell damage was examined morphologically and by vital dye exclusion; respiration, anaerobic glycolysis, nucleic acid and protein synthesis, and lactate dehydrogenase leakage were the biochemical parameters used to monitor cell injury; and growth potential of the heated cells was assessed from population growth curves* in vitro, *and inoculation of cells into host rats.*

The deleterious effect of hyperthermia increased with duration of heating, and cells actively traversing the cell cycle (log phase populations) were more susceptible to damage than cells engaged in intense metabolic activity but with a minimal replication rate (lag phase cultures). In lag phase cultures, entry into exponential growth was delayed following heating, and in log phase cultures multiplication was temporarily or permanently inhibited. Depression of respiration occurred with all heating schedules examined; anaerobic glycolysis was depressed only after prolonged heating (6 *hr*). *Following hyperthermia a percentage of all populations was killed, but except in the case of lag phase cultures heated for* 9 *hr and log phase cultures heated for* 6 *hr, surviving cells recovered biochemically and morphologically and produced lethal tumours in host rats.*

A striking result of hyperthermia was depression of RNA synthesis in the cells, with depression of DNA and protein synthesis on prolonged heating. It is postulated that the effects of hyperthermia may be mediated through an inhibition of RNA synthesis, with consequent disruption of the normal relationships between DNA, RNA and protein synthesis required for cell replication.

INTRODUCTION

IT HAS been known since the time of Busch in 1866 [1] that hyperthermia (temperatures of 40°C and above) can have a selective destructive effect on some types of cancer cell. Since Busch and other early workers (see [2]) reported the beneficial effect of concomitant erysipelas or other severe pyrexial infection in patients with malignant disease, there have been sporadic attempts [2] to take advantage of the finding clinically for the treatment of cancer, including the classic work of the American physician Coley, who, in the early years of this century induced hyperpyrexia in cancer patients by injection of bacterial toxins, and achieved considerable success in the regression of tumours with this technique [3].

In 1927 Westermark first reported the increased sensitivity of respiration and glycolysis in tumours at elevated temperature compared to that of normal tissues [4]. Cavaliere *et al.* [2] demonstrated that at 42°C the damag-

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ing effect on respiration of Novikoff hepatoma cells and Ehrlich ascites carcinoma cells was irreversible, and confirmed that the effect was selective for the cancer cells, respiration in normal and regenerating rat liver cells showing little difference at 42 and 38°C. The potential for exploiting this difference in biochemistry between normal and cancer cells was illustrated by the success achieved by these workers in treating cancers of the limbs by regional perfusion with prewarmed blood to elevate the tumour temperature to the region of 42°C [2]. In further studies, it was found that not only respiration, but the incorporation of labelled precursors into DNA, RNA and protein into cancer cells, was inhibited at elevated temperature [5, 6].

With the advent of more effective and controlled methods of elevating total body temperature in man [7, 8], and recent reports that the action of chemotherapeutic drugs on neoplastic cells can be potentiated by hyperthermia [9, 10], interest in the effects of heat on cancer cells has gained further impetus.

In the present work an *in vitro–in vivo* system has been used to study the effects of elevated temperature on the biochemistry and growth potential of a malignant cell line. Cell damage *in vitro* after hyperthermia was monitored using respiration, anaerobic glycolysis, nucleic acid and protein synthesis, and enzyme leakage as biochemical parameters; growth potential of the heated cells was assessed from population growth curves and inoculation of cells into host rats. There is now considerable evidence that the position of the cancer cell in its generation cycle at the time of exposure governs the response to chemotherapy [11], and to radiotherapy [12], and in this report the influence of the duration of heating and the metabolic state of the cell population (lag phase or logarithmic phase of growth) on the response to hyperthermia have been investigated.

MATERIAL AND METHODS

Cell line

The cell line used in the present work was established by one of us (JAD) in 1964.

The cells were derived from a 7,12 dimethylbenzanthracene-induced adenocarcinoma of the breast in a female Sprague–Dawley rat [13]. After removal, the mammary tumour was disaggregated into a unicellular suspension using a solution of 1% trypsin (Difco 1:250) in Rinaldini saline [14], and a finger-pump apparatus as described by Boyse [15]. After 4 months in monolayer culture, a fibroblast line became established from populations which originally included cells of epithelial morphology, and the line has been maintained on Waymouth medium MB 752/1, supplemented with 10% pooled human AB serum. The cell line was designated "SDB" because of its origin from a Sprague–Dawley breast tumour, and more specifically "SDB QUB" to indicate its laboratory of origin (Queen's University, Belfast), and conform with the nomenclature recommendations of the Tissue Culture Association for new cell lines [16]. Since its establishment the cell line has been tested regularly for the presence of pleuropneumonia-like organisms, and has always been found to be free of this contaminant.

Culture techniques

The cells were maintained routinely in 3¼ inches diameter plastic petri dishes (Esco, grade AA), and for experiments similar petri dishes 2 in. in diameter were used. The initial inoculum into each 2 in. petri dish was $1{\cdot}0 \times 10^5$ cells in 3 ml Waymouth medium with 10% pooled human AB serum. Experimental cultures were always set up from cells in the logarithmic phase of growth (day 3); this gave a plating efficiency consistently in the region of 100%. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air using a CO_2 incubator, and the medium was renewed on day 4. For hyperthermia, cultures were placed for the appropriate length of time in a CO_2 incubator maintained simultaneously at 42°C. Cells were harvested from the petri dishes for subculture or for animal inoculation with 0·1% trypsin (Difco 1:250) in Ca^{2+}— and Mg^{2+}— free Rinaldini saline.

Animal bioassay system

The growth potential of the cells following heating was assessed from subsequent growth curves and biochemical studies *in vitro*, and by inoculation of cells into Sprague–Dawley rats. Several injection sites, including the peritoneal cavity, liver, and brain, were investigated, using cell populations ranging from 1×10^3 to 50×10^6 cells. For liver injection, the right and left central lobes were exteriorized via a midline abdominal incision by gentle pressure on both sides of the lower thoracic cage, and the cells injected under direct vision in 0·1 to 0·2 ml Waymouth medium, using a fine bore (No. 12) needle. Technically successful injection was indicated by blanching at the needle site and lack of extravasation when injecting the suspension, although transient bleeding usually

occurred on withdrawal of the needle from the liver.

Cell counts were made on an improved Neubauer haemocytometer chamber using phase contrast optics. Duplicate counts were made using a fresh chamber for each count, after diluting the suspension to a concentration of about 1×10^6 cells/ml. Viability of the cell populations was assessed using trypan blue [17] at a final concentration of 0·25% in the presence of 5% pooled human AB serum.

Warburg manometry

For respiration studies, an optimal number of cells, 7 to 10×10^6, was suspended in 3 ml Krebs–Ringer phosphate solution, pH 7·4, containing 0·01 M sodium succinate in each Warburg flask. The flask contained air as the gas phase, with 0·2 ml 10% KOH in the centre well, and O_2 uptake was expressed as μl per mg dried trichloroacetic acid (TCA)–insoluble material per hr (QO_2).

For measurements of anaerobic glycolysis, 7 to 10×10^6 cells were placed in Krebs–Ringer bicarbonate buffer, pH 7·4, and the CO_2 production recorded. The gas phase was 95% N_2 and 5% CO_2 (initial O_2 content of mixture less than 20 ppm, Air Products Ltd), and results were expressed as μl CO_2 produced per mg TCA–insoluble dry weight of cells per hr (QCO_2), in the absence of glucose (endogenous glycolysis), and with glucose (2 g/l) added to the bicarbonate buffer. All manometric observations were carried out in duplicate flasks. Lactate determinations indicated that the CO_2 displacement from the bicarbonate buffer was a reliable measure of glycolysis in the cell populations under the various experimental conditions.

Chemical analyses

For nucleic acid, protein and enzyme analysis, the cells were removed from the petri dishes with 0·04% crystalline trypsin (Sigma, bovine pancreas type I, 2 × crystallized) in Rinaldini saline. This enzyme has been reported to cause the release of only 1% of the cellular nucleic acids, with minimal damage to the cell surface, during the harvesting of cultured cells [18].

After preliminary removal of the acid soluble fraction and lipids, DNA in the cells was determined by the method of Burton, and RNA by difference at 260 mμ as detailed in [19]; a solution of pure bovine serum albumin was used as protein standard for the Lowry method [19].

Lactate dehydrogenase in cells and medium was assayed as described in [20]. The cells were kept at 4°C during all manipulations, except spectrophotometry, which was performed at 25°C, and the enzyme measurements were always completed on the same day as each experiment.

RESULTS

Bioassay system

Tumours occurred in all sites tested, but the results were most easily and consistently observed when the liver of weanling animals was used for inoculation. Injection of 10×10^6 cells in 0·1 ml suspension into the liver of 4 to 6-week old rats was adopted as standard procedure for the bioassay. With this number of cells all the experimental animals died with tumour at approximately one lunar month after injection, and the tumour was so widespread throughout the abdomen, with ascites, that the bioassay result could be observed, and palpated to be positive, within 21 days.

The tumours at all sites were anaplastic with pleomorphic cells and numerous mitotic figures (Fig. 1). The overall appearance was that of an undifferentiated, highly malignant tumour with features suggestive of a connective tissue origin.

Heat sensitivity of SDB cells

The sensitivity of the cells to increased temperature was assessed as indicated in Fig. 2. O_2 uptake was higher in cells heated at 39 and 40°C than in control populations maintained at 37°C; at temperatures above 40°C there was a depression of respiration. At 42°C irreversible damage to the cells occurred after 4 hr, as indicated by low O_2 uptake (Fig. 2a), loss of viability (Fig. 2b), and failure of cells to proliferate (Fig. 2c). On the basis of these results, 42·0°C was selected as the lowest elevated temperature which exerted an irreversible inhibitory effect on the respiration and multiplication of the SDB cells.

Effects of hyperthermia (42°C) *on monolayer cultures*

Figure 3 illustrates the population growth curve, and concomitant biochemical events, in control cultures of SDB cells maintained over 7 days at 37°C.

The cells became attached to the Petri dish surface within 4 hr of inoculation, and the lag phase, during which cell multiplication was minimal, lasted a further 20 hr (Fig. 3a). The population then entered exponential multiplication with peak values per cell of

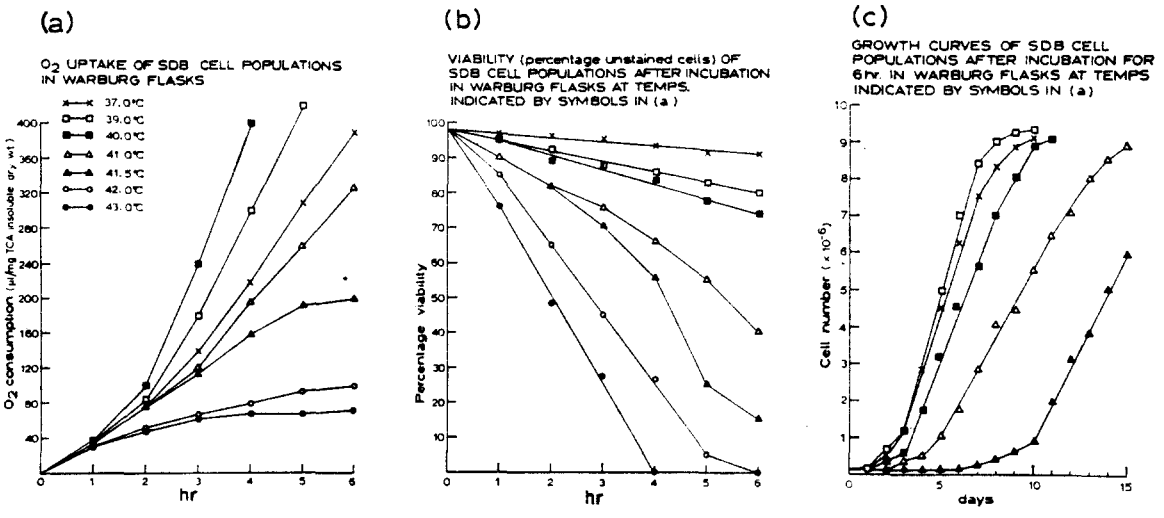

Fig. 2. O_2 *consumption (a) and viability (b) of SDB cells incubated for* 6 *hr in Krebs-Ringer phosphate buffer, pH* 7·4, *containing* 0·01 *M sodium succinate, and then transferred to plastic petri dishes, and the subsequent growth curves in Waymouth medium* MB 752/1 *studied at* 37°C *(c). The Warburg flasks contained* 7–10 x 10^6 *cells initially, and each petri dish was inoculated with* 1 x 10^5 *viable (unstained) cells. The final buffer pH in the flasks was* 7·30 *to* 7·35. *Each point on the graphs is the average value from at least two separate experiments.*

DNAP, RNAP and protein (Fig. 3b), and with maximum levels of glycolysis (Fig. 3c), and remained in log phase until the fourth day, when the rate of cell replication decreased until the cell monlayer reached confluency at day 6.

The effects of hyperthermia on the SDB cells were assessed in terms of the parameters detailed in Fig. 3, in association with alterations in cell morphology and the ability of the heated cells to produce tumours in weanling rats.

Cell populations in the lag phase or the logarithmic phase of the growth curve were heated at 42°C for periods of up to 9 hr. On transferring the cultures from 37 to 42°C, there was a delay of approximately 1 hr before the medium in the culture dishes reached 42° C. A similar delay in equilibration occurred when the cultures were returned to 37°C after heating. All manometric measurements were made at 37°C.

The effects of hyperthermia were more severe

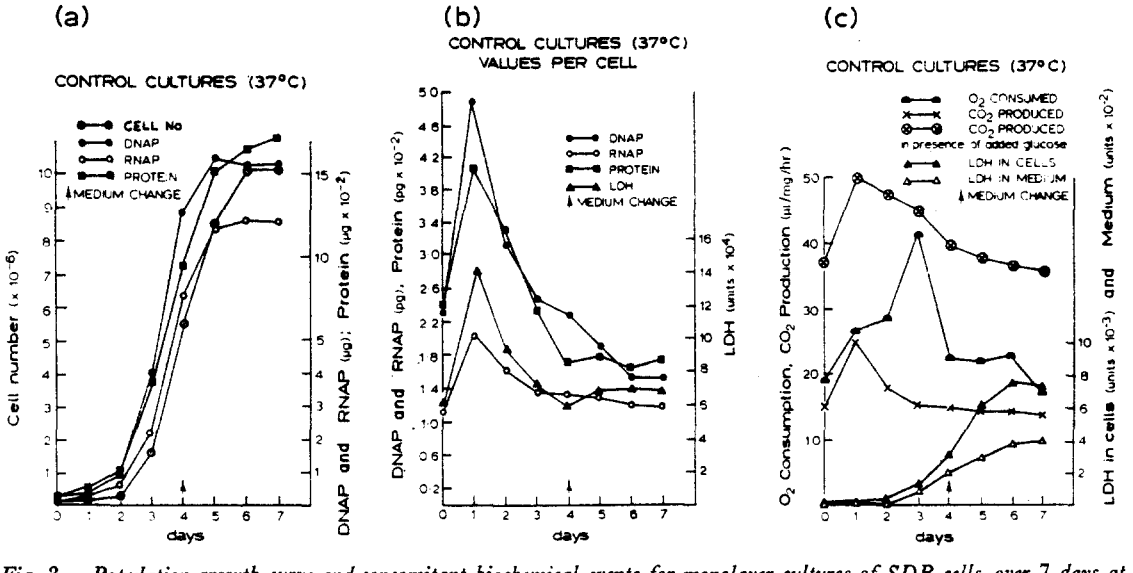

Fig. 3. Population growth curve and concomitant biochemical events for monolayer cultures of SDB cells over 7 *days at* 37°C *in plastic petri dishes. Initial number of cells inoculated into each dish was* 1 x 10^5. *In each experiment the individual points were determined by analysis of cells from* 2 *to* 20 *dishes. The values for the initial* 2 *days of the curves, when the cell numbers were small, were verified by inoculation of* 5 x 10^6 *cells into the dishes and performing the various measurements after harvesting the cultures at* 24 *and* 48 hr. *Each point on the charts represents the average value from* 6 *separate experiments.*

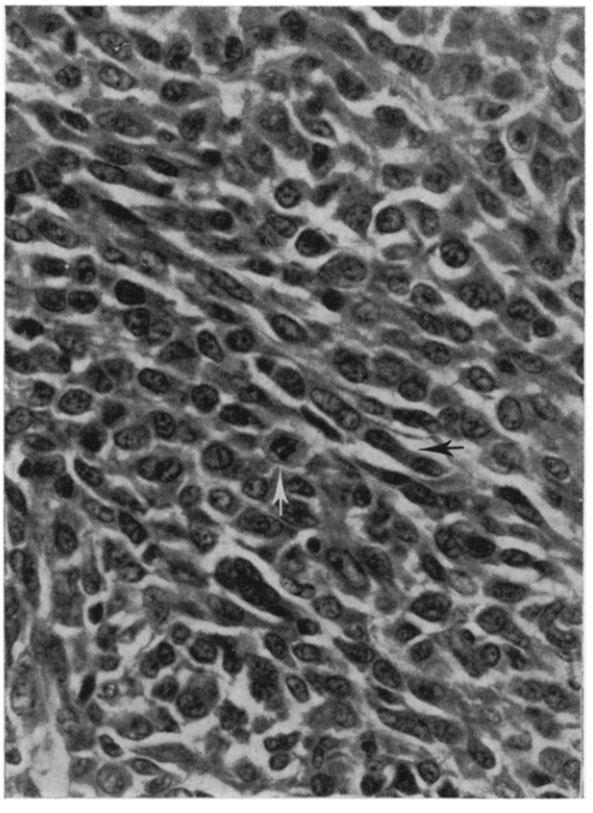

Fig. 1. *Section of tumour in liver of rat which died* 27 *days after injection of* 10×10^6 *SDB cells into liver. The mixed nature of the tumour, with areas of spindle cells interspersed through regions more typical of a carcinoma, is illustrated. The network separating the cells (and shown by differential staining to be reticulin and collagen) is prominent. A spindle cell with two nuclei (black arrow), and a cell in early anaphase (white arrow), are present. Haematoxylin and eosin* (× 800).

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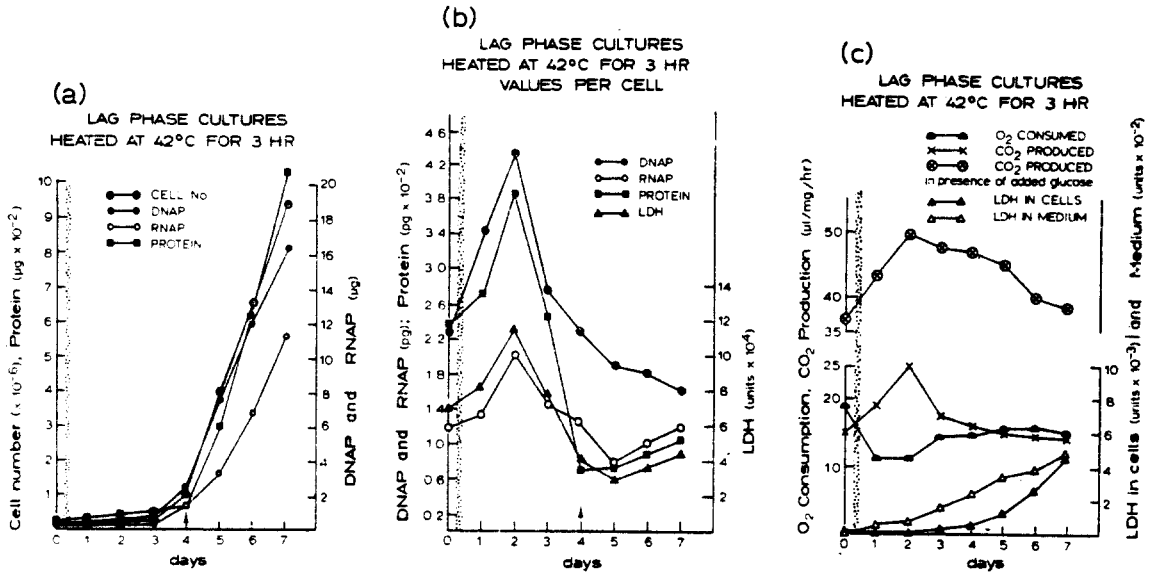

Fig. 4. Population growth curve and concomitant biochemical profiles for monolayer cultures of SDB cells heated at 42°C *for* 3 *hr in the lag phase. Cultures were heated* 6 *hr after introduction of* 1×10^5 *cells into each dish (stippled line). In each experiment the individual points were determined by analysis of cells from* 5 *to* 20 *dishes. When the cell numbers were less than* 1×10^6 *(days* 1–4*), the values were verified by several experiments in which an initial inoculum of* 2×10^6 *cells was used in the dishes.* ↑ *indicates medium change. Each point on the charts represents the average of* 4 *separate experiments.*

on cells heated in the log phase than on cells heated in the lag phase (Figs. 4–7). The percentage of the cell population killed by heat is given in Table 1. The dead lag phase cells became detached from the dish surface within 24 hr. After log phase heating the cultures became detached from the surface, and many cells were reduced to debris; surviving cells reattached to the dish within 24 hr. A considerable number of surviving cells had an abnormal morphology, with elongated attachment processes, and in some cases cytoplasmic and nucleolar vaculation, changes which have been reported by other workers for tissue culture cells exposed to supranormal temperatures [21, 22]. Once replication resumed, however, return to normal morphology was rapid. The increased MGT of the cells after heating (Table 1) returned to control values on subsequent subculture.

The most striking effect of hyperthermia was an inhibition of cell multiplication, so that entry of the population into exponential growth was delayed until day 2 after 3 hr heating

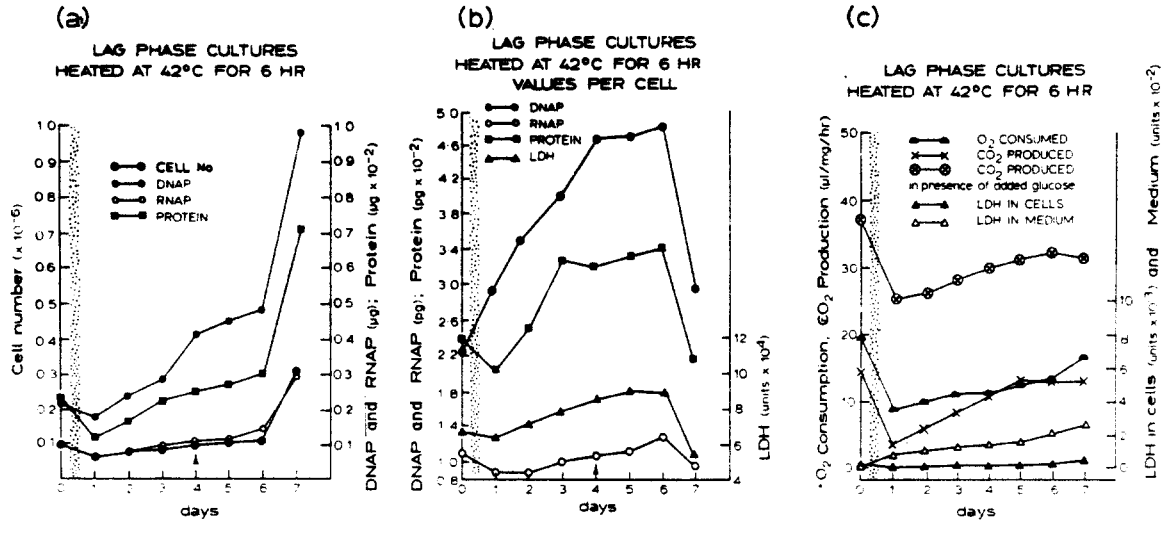

Fig. 5. Population growth curve and biochemical profiles for SDB cultures heated at 42°C *for* 6 *hr in the lag phase. All values were verified using initial inocula of* 2×10^6 *cells per dish. Otherwise legend as for Fig.* 4.

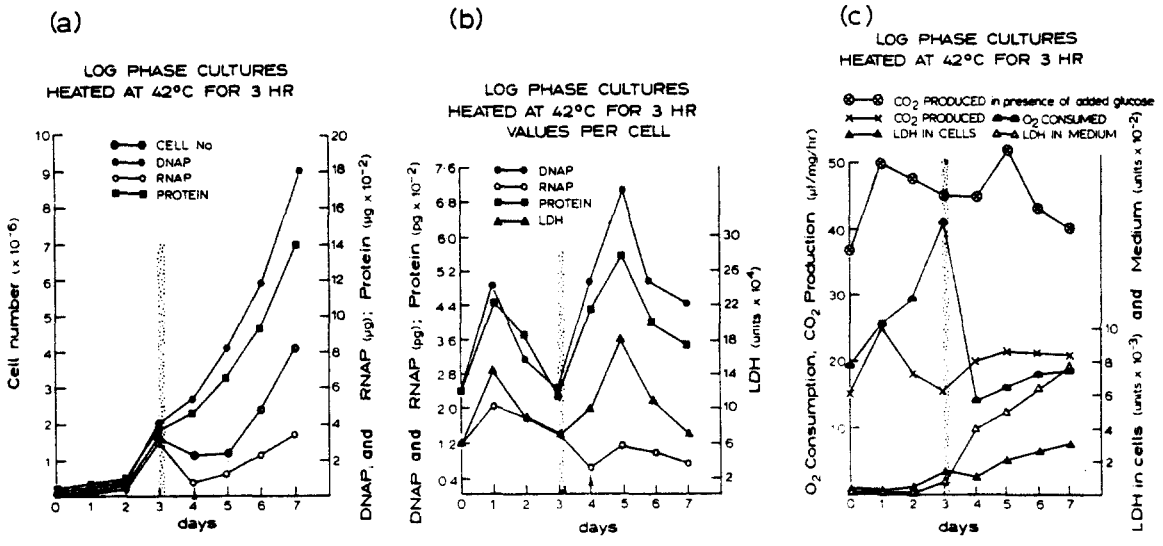

Fig. 6. Population growth curve and biochemical profiles for SDB cultures heated at 42°C *for* 3 *hr in the log phase. Cultures were heated on day* 3 *after inoculation of* 1×10^5 *cells (stippled line). All values over the first* 6 *days were verified using initial inocula of* 1×10^6 *cells per dish. Other details of legend as in Fig.* 4.

(Fig. 4), and until day 6 after 6 hr heating (Fig. 5). Heated log phase cells re-entered a 2-day lag phase (Fig. 6), and after 6 hr at the elevated temperature became almost inactive metabolically (Fig. 7).

As the cultures renewed multiplication, maximum values for DNAP, RNAP and protein per cell compared favourably with values in control populations, with the exception of RNAP, which was 40% lower per cell in 6-hr lag heated cultures, and 3-hr log heated cultures, at entry into exponential growth (Figs. 3, 5 and 6; Table 1); after prolonged log phase heating the cells contained very low levels of RNAP (Fig. 7; Table 1).

By heating small numbers of cells in gridded dishes, plating efficiencies were obtained for the cell populations which survived hyperthermia. Following lag phase heating for 6 hr 5% (maximum) of the cells did not resume multiplication, and after log phase heating for 3 hr the maximum figure was 12%. Account has not been taken of such cells in the figures and tables in this section, but the percentages are too small to significantly alter the results.

Depression of respiration occurred in SDB cells with all heating schedules examined. Anaerobic glycolysis was only affected by prolonged heating (6 hr), and restoration of the glycolytic and respiratory rates toward normal

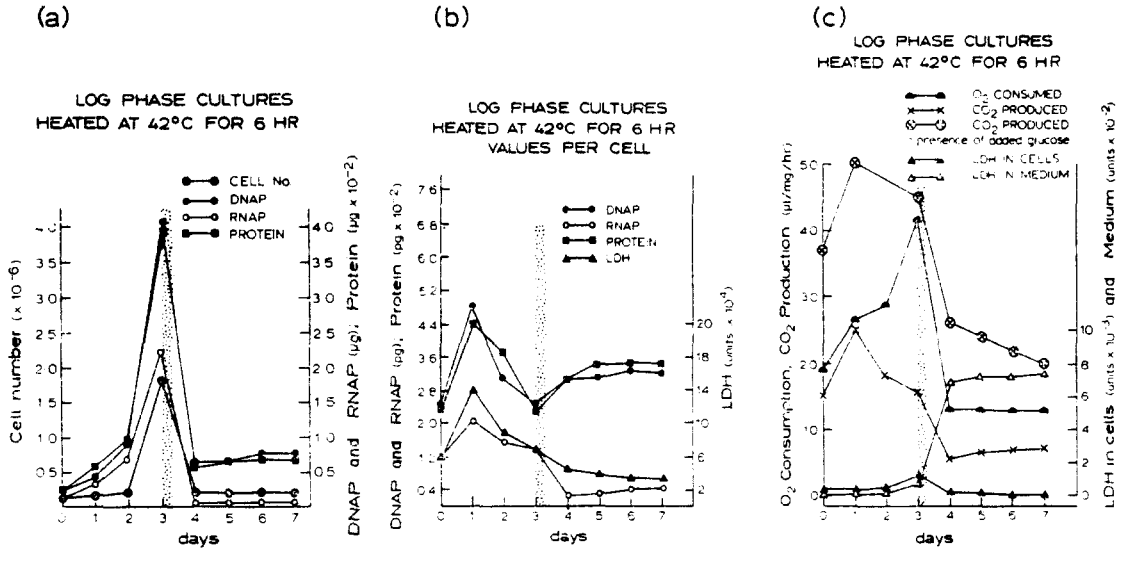

Fig. 7. Population growth curve and biochemical profiles for SDB cultures heated at 42°C *for* 6 *hr in the log phase. Legend details as for Fig.* 6.

Table 1. *Population viability, maximum nucleic acid and protein values attained per cell, and mean generation time (MGT), in SDB cultures following hyperthermia*

	Control 37°C	Cells heated in LAG phase 3 hr	6 hr	Cells heated in LOG phase 3 hr	6 hr
Population viability (%) after heating		80	58	57	5
Day of entry into log phase*	1	2	6	5	—
DNAP (pg)	4·9	4·4	4·8	7·1	3·2
RNAP (pg)	2·1	2·0	1·3	1·2	0·4
Protein (pg)	410	380	340	550	350
MGT	12·0	13·4	18·6	28·8	—

pg = 10^{-12}g.

*Maximum values were attained at day of entry into log phase. Log phase cultures heated for 6 hr did not re-enter exponential growth.

values paralleled the recovery of replicative ability in the cells.

Following heating, there was increased leakage of lactate dehydrogenase per 10^6 cells from all cultures (Figs. 3 – 7). In cells which resumed replication, enzyme levels in the medium rapidly returned to control values, indicating, that, with sublethal heating, the damage to the cells was reversible.

Table 2. *Injection of* 10×10^6 *heated SDB cells into the liver of weanling Sprague–Dawley rats.*

Cells injected	No. of rats dead/injected	Time of Death (days)	Mean time of death (days)
Control	12/12	18–30	26·3 ± 3·7*
3 hr lag	10/10	21–32	27·0 ± 4·2
6 hr lag	10/10	24–32	28·3 ± 4·0
9 hr lag	0/4	—	—
3 hr log	14/14	25–37	30·2 ± 4·4
6 hr log	0/10	—	—

*Mean value ± std. deviation. Rats in the 9 hr lag and 6 hr log groups were observed for up to 2 years following cell inoculation.

There was no statistically significant difference in survival times between the controls and animals which died after injection of heated cells.

On inoculation of the cells immediately after heating into rats, only animals which received 6-hr log heated or 9-hr lag-heated cells survived (Table 2). The 9-hr lag-heated cultures had a viability of 15%, with low QO_2 and QCO_2 values, and after hyperthermia no further multiplication occurred.

DISCUSSION

The three distinct phases of growth, the lag, the logarithmic, and the stationary, and the pattern of DNA, RNA and protein values per cell observed with SDB cells over these phases, have been reported previously for other established cell lines in monolayer culture [23–26]. Incorporation studies with radioactive nucleic acid and protein precursors have confirmed that the levels of cellular DNA, RNA and protein during the various growth phases reflect their rates of synthesis during these periods [25, 27].

The effects of heat were more severe on SDB cells actively traversing the cell cycle, than on intensively metabolizing cells with minimal replication rate (lag phase, which includes predominantly cells in the G1 phase, the S phase or the G2 phase of the cell cycle, with a small number of cells in mitosis). The cells cannot be cultured in a resting G1 state on maintenance medium, and there is evidence that, in a confluent monolayer the metabolism of the cells alters, due to interaction between contiguous cells in the high density population [28]. It has been established by various workers that both normal and malignant cells are most sensitive to an increase in temperature during mitosis, and more specifically in the stage of metaphase (see [2] for refs). In detailed experiments with human amnion cells in culture, Sisken *et al.* [29] found that all phases of the cell cycle were sensitive to temperatures above 39°C, metaphase especially so.

Almost fifty years ago, Westermark [4] reported that the glycolysis, both aerobic and anaerobic, of Jensen sarcoma and Flexner–Jobling carcinoma slices became progressively

reduced with time of heating at 44–49°C, while the respiration remained constant for as long as 90 min, depending on the temperature; finally respiration suddenly ceased, at a time which corresponded to the reduction of glycolysis to negligible levels. The times required for cessation of metabolism in the tumours correlated well with the time of *in vivo* heating necessary to produce recession of the tumour in the host. From his detailed studies on normal and malignant tissues, Westermark concluded that the primary damage in heating was to the enzymes of glycolysis, and that the thermal sensitivity of tissues increased in parallel with their glycolytic capacity [4]. Johnson [30] reported that between 42·5 and 47°C there was a decrease, but not cessation, of glycolysis in slices of Walker 256 carcinoma and Jensen sarcoma; respiration results were not mentioned by this worker, but the heated tumour slices did not grow on transplantation. In more recent work, Rossi-Fanelli and his group found that the respiration of Ehrlich ascites cells, Novikoff and minimal deviation hepatoma cells, was depressed at 42 to 43°C, while anaerobic glycolysis was unaffected or increased [2, 5]. The authors postulated that the damaging effect of heat on tumour cell respiration should be regarded as a general phenomenon, but pointed out that the conditions of heating merited careful consideration in hyperthermia studies, and illustrated this by the difference in manometric measurements made at elevated temperature, and results obtained at 38°C after preincubation at the increased temperature [5]. In heat-susceptible human cancers, respiration and/or glycolysis of tumour slices are depressed after incubation at 42°C [31].

It is possible that the various results reported for the thermal sensitivity of respiration and glycolysis in cancer cells reflect the difference in tumour cell types, in culture conditions (e.g. monlayer vs. suspension cultured cells), and in conditions of heating. It must also be remembered that the conditions under which respiration and glycolysis are usually measured (i.e. Warburg manometry), although convenient, cannot be regarded as physiological [32]. The need for an *in vitro–in vivo* assay system to evaluate the results of Warburg manometry and of biochemical studies, in terms of growth potential of the cells being examined, becomes evident therefore. In the present work, the ability of the SDB cells to multiply *in vitro* following heating paralleled their ability to produce tumours in the rat; only in cells which did not produce tumours *in vivo* (6 hr-log-heated, 9 hr-lag-heated) were respiration and glycolysis irreversibly depressed below control values (Fig. 7). No further *in vitro* multiplication occurred in these cells, and metabolism continued at a low level, a situation reminiscent of X-ray produced "feeder" cells [33]. In recent experiments with the rabbit VX2 carcinoma, tumour cells in which respiration was irreversibly depressed below control values by heating at 42°C did not give rise to tumours in host animals, although anaerobic glycolysis in the cells continued unabated following the heating [34]. On the other hand, Burk *et al.* [35] have emphasized the importance of anaerobic glycolysis in tumour cell biochemistry, and described a positive correlation between *in vitro* glycolytic potential and *in vivo* growth rate in hepatomas. In this context, glycolysis was maximum as the SDB cultures entered exponential growth (Figs. 3–6), and glycolysis in the presence of added glucose was highest during log phase growth. Following heating, the integrated metabolism of the cell is disturbed, and it seems probable that decreases in respiration or glycolysis are secondary changes to earlier and more subtle damage at the RNA level (see later). The initial lesion may also involve lysosomal or surface membranes, as postulated by Mondovi *et al.* [5, 6]. It would seem, however, that irreversible disruption of the replicative machinery of the heated cell renders glycolysis of less significance in metabolism than is usually accepted for cancer cells [35, 36].

Mondovi *et al.* [6] found that at 40°C and above the incorporation of labelled thymidine, uridine and amino acids into the nucleic acids and protein of Novikoff and hepatoma 5123 cells was progressively decreased and ultimately inhibited, depending upon the temperature and duration of the experiments, while the incorporation of similar labelled precursors into regenerating liver cells was higher at 43 than at 38°C. In the present experiments RNA synthesis was more severely affected by hyperthermia than DNA or protein synthesis. An effect on DNA and protein synthesis was apparent only after 6 hr heating of log phase cells; even then inhibition was not detected until 2 days after heating (Fig. 7). With RNA, on the other hand, a rapid decrease in synthesis occurred following hyperthermia, with a subsequent recovery to levels per cell markedly below that usually present when the cells entered exponential growth (Figs. 3, 5, 6; Table 1). When the peak values of DNAP, RNAP and protein per cell at entry into exponential multiplication are considered (Figs. 3–6; Table 1), it is seen that all three parameters

reached a maximum value simultaneously on entry into log phase, but in Figs. 5, 6, in cells which survived to replicate following severe damage by heating, both DNAP and protein per cell had achieved high values 24 to 48 hr before the population entered log phase; the values achieved were previously high enough for entry into log phase. It is therefore tempting to infer that the RNA level in the cell is important in controlling entry into cell division. Such a proposal is in keeping with current knowledge of the biochemical events controlling cell replication, and with results obtained at the ultrastructural level. It is now recognized that the cell cycle is based upon a temporal series of genetic transcriptions, which remain in sequence by a succession of dependencies of one transcription upon the next [37]. Mueller and Kajiwara [38] have shown that the properly-timed synthesis of RNA and protein is essential for the production of late-replicating DNA in HeLa cells, and for the mitotic process. When RNA synthesis is blocked at the beginning of DNA synthesis, the cells replicate only 55 to 65% of their DNA, and fail to progress into mitosis unless RNA synthesis is allowed to resume, and the cells complete replication of their DNA [39]. There is also a requirement for the synthesis of some RNA late in S phase; if this RNA is not synthesized, again the cells fail to enter mitosis [39]. In detailed studies on RNA metabolism in HeLa cells maintained at 42°C in suspension culture, Warocquier and Scherrer [40] found that RNA synthesis in general was depressed. Ribosome synthesis was selectively inhibited, and the gradient pattern and base composition analyses were consonant with the primary lesion being defective conversion of 45 S pre-ribosomal RNA to functional 28 S and 18 S species. Proliferating cells would therefore be more susceptible to damage at elevated temperature than non-proliferating cells [40].

From high-resolution EM autoradiography studies, Simard and Bernhard [41] reported striking nucleolar lesions in cultured BHK cells after exposure to 42 to 45°C; the granular RNP disappeared, and augmentation and condensation of the fibrillar RNP occurred. It was pointed out that these results correlated with the defective transformation of 45 S RNA into 28 S and 18 S fractions, which is believed to take place sequentially via the fibrillar and granular components of the intranucleolar chromatin [41]. Subsequently, several correlative ultrastructural and biochemical studies on different cell types have confirmed the occurrence of these nucleolar lesions at supranormal temperatures, with associated defective synthesis and metabolism of the 45 S ribosome precursor RNA [42–44]; the complete reversibility of the changes after periods of hyperthermia extending up to 3 hr with some cell types, has also been corroborated [43, 44]. McCormick and Penman [45] reported that in HeLa cells incubated at 42°C, the rate of amino acid incorporation decreased precipitously within 10 min, and extensive polyribosome disaggregation occurred. On further incubation at this temperature, there was a partial recovery of the translation process, with an increase in the uptake of amino acids and in the number of functioning ribosomes. This recovery was dependent upon RNA synthesis, but not protein synthesis [45]. Polysome disaggregation in heated cells, with polysome reassembly following up to 3 hr heating, has been reported by other workers [43, 44].

The present results indicate that RNA synthesis in SDB cells is sensitive to elevated temperature, and the inhibitory effect of hyperthermia on cell replication may be mediated by the consequent dissociation of the relationship between DNA, RNA and protein synthesis required for cell replication. Precise localization of the lesion caused by hyperthermia, in terms of the temporal sequence of transcriptions envisaged by Prescott [37], and characterized biochemically for HeLa cells by Mueller [39], will require more information on cell cycle events at elevated temperature, since there is evidence from studies on human amnion cells that the usual relationships between the biosynthetic processes of the cycle are altered at high temperatures [29].

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Investigations on the Possibility of a Thermic Tumour Therapy—II

Action of Combined Heat-Roentgen Treatment on a Transplanted Mouse Mammary Carcinoma*

KRISTIAN OVERGAARD and JENS OVERGAARD

Cancer Research Institute, Danish Cancer Society, Radiumstationen, DK 8000 Aarhus C, Denmark, and Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK 8000 Aarhus C, Denmark

IN A PREVIOUS study, we demonstrated that local heat application may exert an elective destructive effect on malignant tumour tissue[1].

The necessary relations between temperature and exposure time for an anaplastic, isologous mammary mouse tumour were worked out, and the special histological findings were described.

Further investigations on the above-mentioned tumour have revealed a highly significant intensification of the tumour-deleting effect obtained by the addition of a small dose of local roentgen irradiation.

By successive application by a heat dose and a roentgen dose, both substantially smaller than the lowest curative doses, a large number of total cures may be obtained (Table 1).

The curative effect depends on the size of both the heat dose and the roentgen dose.

The effect is largely unaffected by the sequence of the two components, and an interval of up to 24 hr between the applications does not alter the curative effect appreciably.

The histological reactions after combined treatment differ distinctly from those observed after either heat or roentgen application separately: The resting and less oxygenated parts of the tumour tissue succumb rapidly in a totally necrotic mass without any signs of new tumour growth.

The marginal, better oxygenated and active tumour cells show a highly variable and protracted process of decay in successful cases with final, complete deletion of the tumour cells and, in about 6 to 8 weeks, the formation of a fibrotic scar.

If insufficient doses are applied, a few surviving tumour cells may cause new tumour growth in this region.

The effect is clearly confined to the tumour cells, leaving all normal cells undamaged. Only some normal cells placed in special relation to the tumour cells (stroma, some marginal tissue, etc.) may secondarily show signs of affection.

Just as in the case of heat treatment alone, release and activation of lysosomal acid hydrolases in the tumour tissue is the most likely explanation of the destructive action of the combined heat–roentgen treatment. After this treatment, measurements of lysosomal enzymes initially show increased activity of the entire tumour homogenate, decreasing to normal or subnormal values within the next 48 hr. A similar, although less pronounced, pattern is seen after heat treatment, while no significant changes are observed during the first 48 hr after roentgen treatment (Fig. 1).

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Table 1.

No. of animals surviving after 1 week	Heat (local)		Roentgen (local)	Cures	%
11	0		3200 r	1	9
42	0		2400 r	2	5
98	0		1600 r	0	
40	42·5°/ 60′		0	9	22
20	42·5°/ 30′		0	1	5
21	41·5°/240′		0	8	38
21	41·5°/120′		0	0	
5	41° /480′		0	2	
17	42·5°/ 60′	+	1600 r	13	77
22	42·5°/ 15′	+	800 r	8	35
21	41·5°/ 30′	+	400 r	1	5
73	41·5°/ 30′	+	800 r	6	8
32	41° / 60′	+	1200 r	8	25
10	40·5°/180′	+	1600 r	1	10

300 Untreated controls all died with tumour.
Cures controlled for at least 6 months.
Interval between application of treatments ½ to 4 hr.

The roentgen effect may differ from the mechanism of action usually assumed in therapeutic application. No mitotic activity at all is present, and the very rapid and impressive alteration in the morphological appearance after roentgen irradiation (Figs. 2 and 3) is strongly suggestive of some alteration in the enzymatic processes in the tumour cells.

It is doubtful whether the increased amounts of acid phosphatase and cathepsin observed after the supplementary roentgen dose are released directly by the irradiation.

A rise in the acidity in the tumour cells may secondarily provoke a higher release and also provide more favourable conditions for the activity of the lysosomal enzymes. Furthermore, it may explain the mode of action by the aforementioned variations in the sequence of and the interval between the two components of treatment.

The effect obtained is possibly due to some ability of the roentgen rays to alter the respiratory activity of the tumour cells, as reported by Crabtree [2, 3]. Some alterations observed in the reaction of succinate dehydrogenase may support this view. Any deficiency in the respiratory activity may influence the intracellular acidity and hence the enzymatic activity.

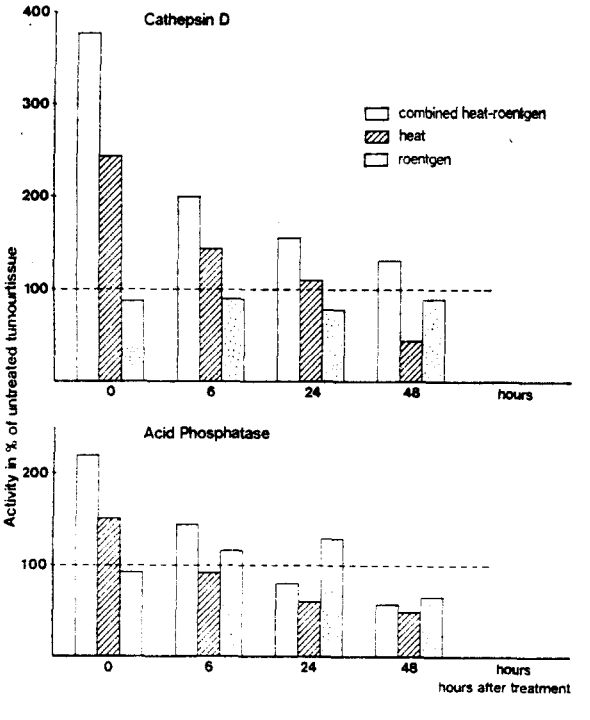

Fig. 1. *Activity of some lysosomal enzymes (acid phosphatase, cathepsin D) after treatment with roentgen* (800 r), *heat* (42·5°C/30′) *or heat and roentgen combined* (42·5°C/30′+800 r, *interval* 3 *hr*). *The activity was measured in the entire tumour homogenate* (% *of untreated controls*).

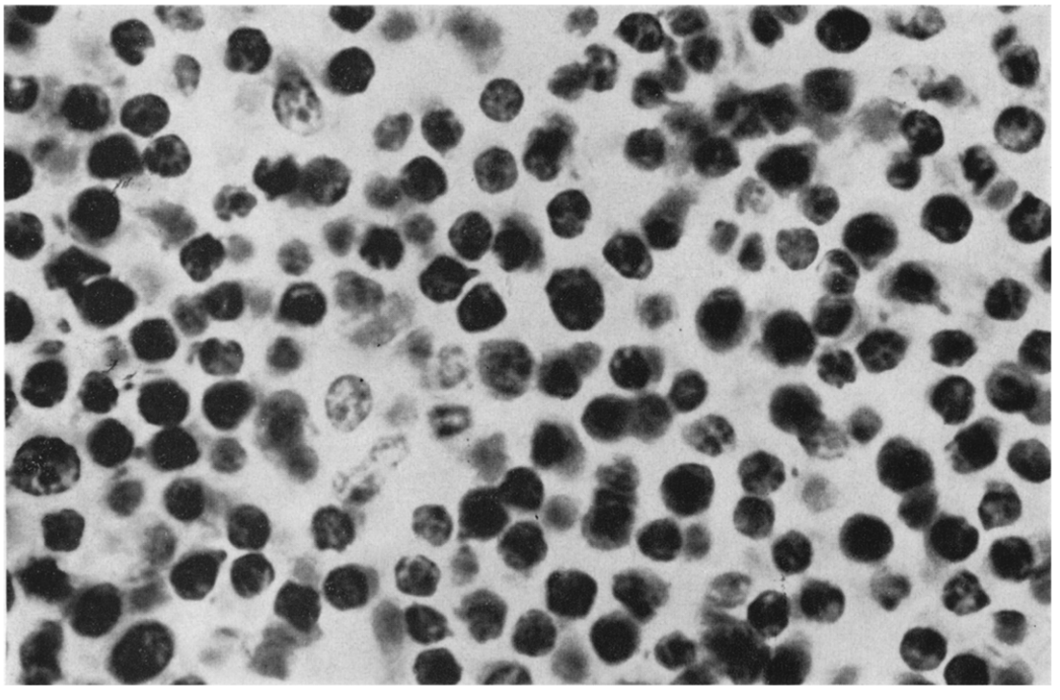

Fig. 2. *Appearance of tumour tissue at* 24 *hr after treatment with local heat* (42·5°C *for* 30 *min*). *The tumour cells show pronounced shrinkage, and the nuclei have also shrunk. In most of the cells, the chromatin has condensed into a single lump. The cytoplasm is visible as a narrow fringe around the nuclei.* 1500×.

(to face p. 574)

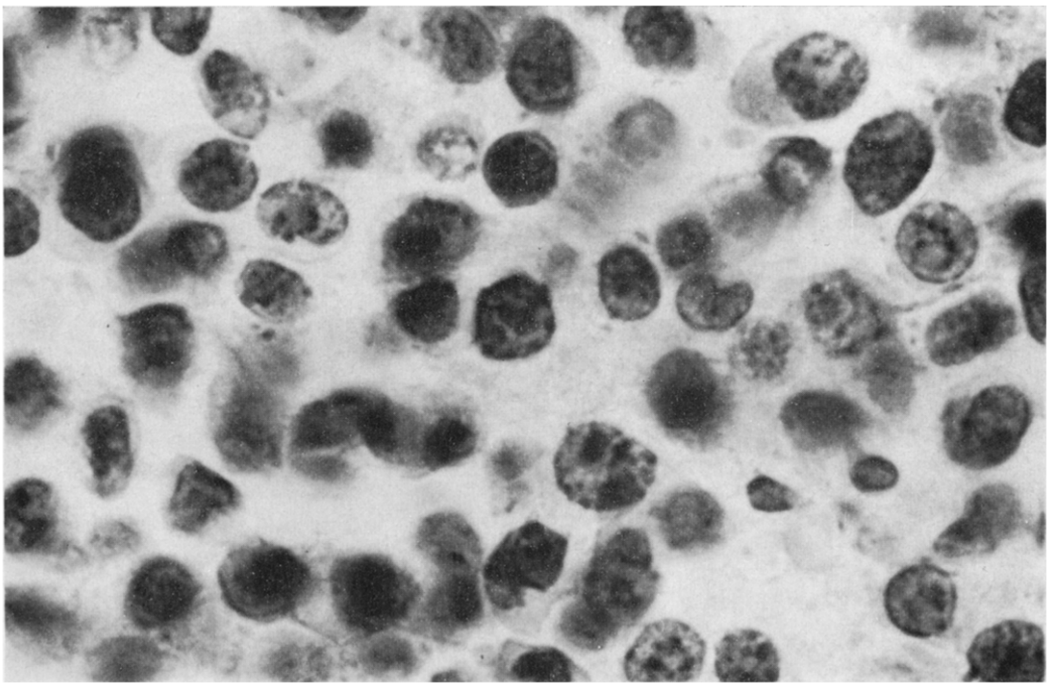

Fig. 3. *Tumour tissue exposed to identical heat treatment.* 24 *hr later supplementary local roentgen treatment,* 800 r, *was given. At* 30 *min after roentgen exposure, the nuclei were swollen, and the chromatin had separated into multiple small clots. The cytoplasmic volume is partially restored.* 1500 ×.

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Letter to the Editor

Density Distribution Spectra of Cell Population of Ehrlich Ascites Carcinoma in Different Stages of Growth

L. HOLCZINGER
Oncopathological Research Institute, Budapest, Hungary

DENSITY distribution analysis is a convenient method of characterizing different cell populations. Experiments with mammalian cells isolated from different organs showed considerable heterogeneity in cell populations [1, 2] as well as an age dependent change of density e.g. in the case of erythrocytes [3, 4]. On the other hand, Anderson *et al.* [5] reported on the density invariance of cultured Chinese hamster cells. Considerable heterogeneity of tumor cell populations was also demonstrated [6–9].

This paper deals with the isopycnic density distribution of Ehrlich ascites carcinoma cell population at various intervals after transplantation.

Swiss mice bearing Ehrlich ascites tumors were sacrificed after 7, 10 or 14 days respectively, after inoculation. After each sacrifice the ascites of 3 animals were pooled and centrifuged in a Christ refrigerated centrifuge at 2500 rev/min at 4°C for 10 min. A linear Ficoll gradient in Hanks' salt solution of which the density varied from 1·02 to 1·13 g/ml was introduced into an MSE B-XIV aluminium zonal rotor rotating at 3000 rev/min in an MSE "Superspeed 50" ultracentrifuge. 2×10^{10} tumor cells were suspended in a high density Ficoll solution (1·13 g/ml) and were introduced at the bottom of the gradient to approach equilibrium from below. A 1·16 g/ml Ficoll gradient was applied as a cushion. The centrifugation was carried out at 25000 rev/min for 90 min at 4°C after which the rotor was slowed down to 3000 rev/min and the gradient was pumped out with a heavy sucrose solution (1·30 g/ml). Twenty millilitre fractions were collected and the viable cell count and the refractive index of each fraction were determined. In one series of experiments the transplantability of cells found at different densities was also investigated.

The isopycnic density distribution profiles of Ehrlich ascites carcinoma are shown in Fig. 1.

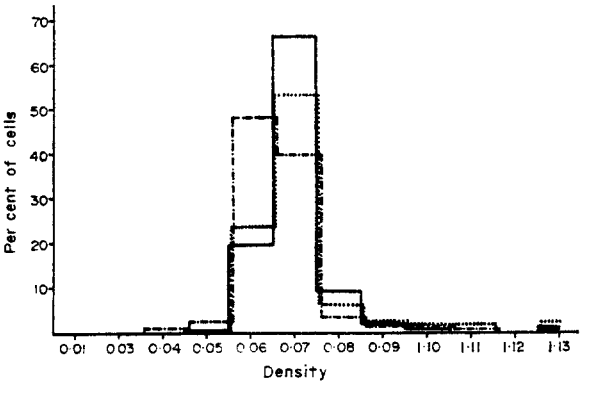

Fig. 1. Density distribution profiles of Ehrlich ascites carcinoma cell population in Ficoll gradients. ——— *7 days* ..... *10 days* _._._ *14 days after transplantation.*

The densities of cells of 7-day-old tumors have a considerably wide range: 1·045–1·105. The distribution profile is characterized by three major zones (1·055–1·065, 1·065–1·075 and 1·075–1·085), where about 90% of the total cell population could be found. The highest percentage of cells (32% of the total) appeared at 1·070. The remaining cells are more or less evenly distributed in the other gradient fractions.

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In 10-day-old tumors, cells were found in gradient fractions ranging from 1·055 to 1·115. The distribution spectrum indicates a slight increase of heterogeneity as compared to the 7-day-old tumors characterized by the appearance of heavy cells (density region 1·105–1·115), as well as by a percentage increase of cells in the 1·055–1·065 density region. The highest percentage of cells (32% of the total) was banded at density 1·067.

The cells of 14-day-old tumors showed the greatest density variations. The densities of cells varied between 1·035 and 1·115, with a peak (30% of the total) at 1·063. More than 50% of the total cell mass appeared between 1·035–1·065, which is more than a two-fold increase in that low density region as compared to the 7 and 10-day-old tumors.

The increased heterogeneity and the gradual shift of the distribution curves toward the lower density regions seems to be a characteristic phenomenon which runs parallel with the ageing of tumor cell population.

Pretlow and Boone [10] have reported a mean density of 1·071 for Ehrlich ascites tumor cells. According to our determinations mean densities for 7, 10 and 14-day-old tumors, calculated on the basis of refractive indices actually determined in each fraction, were 1·0714, 1·0734 and 1·0723 respectively, without correction for osmotic effects of the Ficoll gradient. The variations indicate that mean density is not a permanent parameter which could characterize a tumor in all stages of its growth.

The cells of 7 and 10-day-old tumors, found at densities 1·062, 1·072, 1·078 and 1·088 respectively, could successfully be transplanted, indicating that during the separation process the cells did not lose their ability to induce tumors.

Further investigations are needed however, to determine whether the tumors originating from these subfractions are biologically identical to the original, unseparated tumors, whether they represent distinct cell populations with different biological activity.

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Letter to the Editor

Some Immunological Properties of Canine Prolactin

P. G. SALUJA and H. G. KWA

Department of Experimental Pathology and Cancer Research, University of Leeds, Leeds, England and Antoni van Leeuwenhoek-huis, The Netherlands Cancer Institute, Amsterdam, The Netherlands

PROLACTIN is involved in mammary gland tumorigenesis in mice and rats [1, 2]. It is also implicated in conditions of galactorrhoea in man [3].

In the bitch, spontaneous mammary tumours and non-puerperal lactation occur quite commonly. Breast tumours, which have many similarities to those of man [4], represent approximately 25% of all malignant neoplasms in the bitch [5], while lactation frequently occurs during pseudopregnancy [6].

It would be of interest to have a radioimmunoassay for canine prolactin in order to obtain quantitative information on pituitary and serum prolactin levels in various physiological states and in mammary tumour conditions. This may indicate whether or not prolactin contributes to mammary gland tumorigenesis and galactorrhoea in this species.

To establish a radioimmunoassay, one would ideally use the relevant hormone from the same species for radioactive labelling, as a reference standard and for raising the isologous antiserum. However, if this hormone is difficult to obtain, it may be possible to base the radioimmunoassay on a heterologous system wherein the same hormone, from another species whence it is readily available, competes on equal basis with the test hormone for binding the antibodies raised against the purified hormone of a third species.

In view of difficulties experienced in obtaining dog pituitaries, a study has been made to check for cross-reactivity of canine prolactin with that of other species whose pituitaries are easier to acquire.

The method of Kwa *et al.* [7] was used for isolating prolactin from 0·9 g of female canine pituitary. Approximately 1 g of bovine adenohypophysis was used as a methodological control since highly purified prolactin may be obtained from this tissue by the above method [7]. The bovine and canine glands were processed in exactly the same way, i.e. the granular fraction was isolated by differential centrifugation, subjected to extraction with physiological saline to leach out the growth hormone and finally extracted with sodium carbonate at pH 10 in order to remove the prolactin.

In order to check for hormone loss and contaminations, aliquots of supernatants were subjected to polyacrylamide gel electrophoresis [8] at three stages in the procedure: [1] after sedimentation of the granular fraction, [2] after saline extraction of the granules, [3] after carbonate extraction of the granules. Normal human serum was also electrophoresed as a reference. The results are shown in Fig. 1. It may be seen that the procedure yielded essentially pure preparations of bovine growth hormone and prolactin. The canine adenohypophysis did not behave in exactly the same way. Saline extraction failed to leach all the growth hormone from the granules and consequently a contaminating band of growth hormone appeared in the carbonate extract. The canine prolactin migrated towards the

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Letter to the Editor

Evidence Against the Neoplastic Nature of Virus-induced Erythroblastosis in Mice

P. BENTVELZEN

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

THE FRIEND and Rauscher murine leukaemia viruses induce in various mouse strains a rapidly developing erythroblastosis, characterized by splenomegaly and an excess of nucleated red cells in the peripheral blood [1, 2]. We have raised doubts, however, about the neoplastic nature of this disease [3, 4]. We failed to achieve transplantation of the leukaemic cells as investigated with chromosomal markers: the resulting leukaemia proved to have the recipients karyotype [3]. Furthermore, we observed the erythroblastosis-inducing principle of the Rauscher virus to be defective and we suggested that the helper virus was needed for the spread of the erythroblastosis virus [4]. This would mean that the disease was due to spread of virus and not to proliferation of neoplastically transformed cells.

Thomson and Axelrad [5] devised a method which claimed to detect true tumour cells in Friend virus-infected spleens. Infected cells from a susceptible strain are inoculated into histocompatible hybrids, which are resistant to the virus. The colonies which developed after a week were then assumed to be transplanted tumour cells and not to be induced by the virus present in the inoculum in view of the resistance of the hybrid. This method would also work for the Rauscher virus (H. J. Seidel, personal communication).

We therefore used this method to see whether true neoplastic cells are present in Rauscher virus-infected mice. The mouse strain chosen for this investigation is CBA and the (C57BL × CBA)F_1 hybrid.

In Table 1 are presented the results of titration of a 10%-Rauscher-virus (RLV) preparation injected intravenously into 10-week old mice, according to the spleen-focus assay of Pluznik and Sachs [6]. The CBA strain proves to be fairly susceptible as compared to its F_1-hybrid with C57BL. The latter strain has not been tested in this particular experiment but from various other experiments we concluded it to be about 10 times less susceptible than CBA.

Table 1. Spleen focus assay of Rauscher leukaemia virus

	Average number of foci ± standard error		
Dilution	BALB/c	CBA	(C57BL × CBA)F1
10 ×	confluent	confluent	43·7 ± 4·8
50 ×	confluent	24·8 ± 1·9	9·5 ± 1·8
100 ×	27·6 ± 2·1	10·4 ± 1·6	3·2 ± 0·6
500 ×	3·6 ± 0·4	3·7 ± 0·5	0·4
1000 ×	0·6	1·1	0·3

CBA mice, 8 weeks of age, were inoculated intraperitoneally with 0·1 ml of the RLV preparation. One week later 5 mice were killed, the spleens taken and pooled, and a monocellular suspension prepared. Twenty (C57BL × CBA)F_1 mice were inoculated intravenously with 10^6 cells. Of a part of the suspension a cell-free extract (10%) was made using a Sorvall Omnimixer homogenizer. The intravenous inoculum of this homogenate corresponded to 10^6 cells per mouse.

Five millilitres of the suspension was irradiated with a Philips–Müller X-ray machine (300 kV, 10 mA, HVL 3 mm Cu) at a dose rate of 110 rad/min with a total dose of 3000 rads and the 10^6 cells were inoculated into 20 hybrid recipients.

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Table 2. Colony assay of Rauscher-virus included leukaemia cells of CBA mice in (C57BL × CBA)F1 recipients

	Average number of spleen colonies ± standard error		
	1 week after virus inocculation	2 weeks after virus inocculation	3 weeks after virus inocculation
10^6 living cells	87·2 ± 6·5	51·1 ± 2·4	64·6 ± 4·6
Cell-free homogenate	11·4 ± 1·3	0	7·7 ± 1·8
10^6 irradiated cells	52·7 ± 2·5	22·9 ± 3·1	26·4 ± 2·5

One week later the mice were killed, the spleens taken and fixed in Bouin's fluid and some hours later foci counted with naked eye.

The experiment was repeated with mice which were inoculated with virus two weeks earlier. With mice, which were infected three weeks before, the experiment was modified in that the cells were now given 6000 rads. In this case the cell suspension was agitated by a shaker and by a flow of humidified air, which was led over the surface of the suspension in order to obtain complete aerobic irradiation conditions.

If one compares in all three experiments (Table 2) the results of the intact cells with those of the cell free material, one would conclude that the colonies were mostly due to the transplantation of intact tumour cells, in view of the low numbers with the cellfree material. However, this is contradicted by the relatively high yield of colonies found after irradiation of the cells. A 3000 rads (anoxic conditions) is sufficient to sterilize 10^8 cells of various murine leukaemias tested in this laboratory (J. J. Broerse, personal communication). Furthermore, a whole body radiation with 500 rads of mice made leukaemic by RLV is sufficient to control the disease for several months.

In our view, the colonies are due to virus present in the irradiated cells. As has been found for the Gross leukaemia virus [7] intact cells seem to provide good protection for the infectivity of the Rauscher virus. These results strengthen us in our opinion that the Rauscher disease (and most likely also the Friend disease) is due to accumulation of physiologically abnormal, non-dividing cells. The resultant lack of normal red cells leads to continuous erythropoietic stimulation and in this way to the perpetuation of the disease.

It is not excluded, however, that occasionally some cells become neoplastically converted as continuous erythroblastic cell lines have been developed in the case of the Friends virus [8] and Rauscher virus (D. H. Pluznik, personal communication). Such neoplastic cells do not seem to cause the early arising splenomegaly, however.

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Announcement

A 2nd International Meeting on:

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

will be held at the Radiobiological Institute TNO, Rijswijk (ZH), The Netherlands, on October 3, 4 and 5, 1973.

The purpose of this meeting is first to review progress made since the previous meeting held at Rijswijk in June 1970, with respect to fast neutron production, collimation of neutron beams and analysis of biological effects, whereby attention will also be given to a comparison with characteristics of π^--meson beams. The main part of the discussions however, will be devoted to an evaluation of the data obtained from experiments with normal tissues and tumours in animals and from treatments of tumours in patients, with a view of making recommendations about the types of cancers on which attention should be focused in order to achieve possible improvements in local and regional control through application of fast neutrons and π^--mesons.

The meeting can be attended by all who are actively engaged in the research and planning connected with the subjects described. Presentation of papers will be by invitation only, except that a limited part of the time may be allotted to short communications to be selected by the organizing committee about new special developments and results.

Registration forms and further information can be obtained from Mrs. R. Purmer, Radiobiological Institute TNO, 151 Lange Kleiweg, Rijswijk (ZH), The Netherlands.

G. W. Barendsen and J. J. Broerse

Co-Chairmen of the Meeting

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Specific Induction of Bladder Cancer in Mice by Butyl-(4-hydroxybutyl)-nitrosamine and the Effects of Hormonal Modifications on the Sex Difference in Response

J. S. BERTRAM* and A. W. CRAIG

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England

Abstract—*Butyl-(4-hydroxybutyl) nitrosamine (BBN) has been shown to be a highly potent and specific bladder carcinogen when administered continuously in the drinking water to 50 male and 50 female C57BL/6 mice. Bladder carcinomas were found in all mice reaching autopsy, other toxic effects were absent. Metastatic spread had occurred in 13 mice. In spite of an identical dosage of 35 mg/kg/day BBN, males developed bladder tumours significantly earlier than did females (190 and 253 days respectively). Early changes in the bladder epithelium were also delayed in the female. This sex difference in susceptibility to tumour induction can be abolished by castrating males or conversely by treating females with testosterone. Testosterone restores the tumour-induction time in castrated males to control values. Phenobarbitone, when given concurrently with the carcinogen, had no effect on the induction of tumours by BBN but partially protected mice against the carcinogenic effects of dibutylnitrosamine.*

INTRODUCTION

THE ORGAN specificity exhibited by the carcinogenic N-nitrosamines has been the subject of much investigation since the activity of this class of compound was first demonstrated by Magee and Barnes [1]. Much of the information on organ specificity can be attributed to Druckrey and his colleagues who have published a most comprehensive report of the activity of 65 N-nitro compounds in their BD strain of rat [2]. An intriguing finding by these workers was that of all the compounds investigated only two, namely dibutylnitrosamine (DBN) and its hydroxylated derivative, butyl-(4-hydroxybutyl)nitrosamine (BBN), induced tumours of the bladder [3]. In view of the wide interest in the nitrosamines both as experimental carcinogens and as possible environmental carcinogens to be found in human and animal foodstuffs [4], and also because of the necessity to further understand the aetiology of bladder carcinogenesis, the activity of these two compounds has been further investigated in these laboratories. The C57BL/6 mouse has been exclusively used as an experimental animal because of the large numbers that may be conveniently employed, and the low spontaneous tumour incidence of this strain. The carcino-

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*Present address: McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706.

Abbreviations: BBN, butyl-(4-hydroxybutyl)-nitrosamine; DBN, dibutylnitrosamine.

genic activity of DBN in this system has already been reported [5]. This paper describes the effects of BBN at 2 dose levels in male and female mice. As the experiment progressed it was found that in agreement with our previous results with DBN, male mice were significantly more susceptible to bladder carcinogenesis induced by BBN. Since such sex linked differences have not previously been so clearly demonstrated for the nitrosamines, the effects of sex hormonal and metabolic alterations on the carcinogenic response to DBN and BBN have been investigated.

MATERIAL AND METHODS

Carcinogens

BBN was synthesized by the method of Druckrey *et al.* [3]. The following modifications were found necessary in order to obtain acceptable yields:

(*a*) Commercial 4-chloro-1-butanol (Schuchardt, Munich), only 60% pure as obtained, was first fractionally distilled using a 60 cm Dufton column. The distillation was monitored by gas-liquid chromatography on a column of 5% SE 30 on AW-DMCS Chromasorb W (Varian U.K. Ltd., Manchester).

(*b*) Complete reaction of 4-chloro-1-butanol with n-butylamine (British Drug Houses Ltd.) required reflux for 12 hr.

(*c*) Hydrolysis of the nitrite ester formed after nitrosation of butyl-(4-hydroxybutyl)amine required treatment with 5N HCl at 40°C for 3 hours.

The product was finally fractionally distilled (b.p. 142°C, 0·08 mmHg) and its purity checked by gas-liquid chromatography (15% XF 1150 on Universal B, Phase-Sep Ltd., Queensferry, Flintshire) and by thin-layer chromatography on silica gel using the solvent system hexane, ether, dichloromethane 4 : 3 : 2 [6].

DBN was obtained from Eastman-Kodak, Liverpool and was of high purity.

Animals and carcinogen treatment

Group A. BBN was administered as a 0·022% solution in the drinking water to a total of 100 (50 males, 50 females) inbred C57BL/6 mice housed in groups of 10. Treatment was commenced when 11 to 13 weeks old and was continued until death. Mean body weights when commencing treatment were 28 and 21 g for males and females respectively. Drinking water solutions were changed twice weekly and the volume drunk recorded. Mice were weighed every 2 weeks and the consumption for each mouse calculated on the assumption that individual mice within a cage consumed similar amounts of water [7]. The concentration of BBN in the drinking water was calculated from preliminary experiments to give the same dose of carcinogen (30 mg/kg/day) as consumed by the mice previously treated with DBN [5]. During the time tumours were appearing, mice were examined twice daily, were removed when moribund whenever possible and subjected to a complete autopsy. Tissues were fixed in formol saline and stained with haematoxylin and eosin for histological examination.

Group B. In order to study the effect of a higher concentration of BBN, a group of 40 mice (20 males, 20 females) were given a drinking water solution containing 0·1% BBN. For 20 weeks after starting treatment one male and one female were killed every 2 weeks and a full range of tissues taken for histology. Bladders were distended with formol saline by urethral cannulation before removal [8]. Treatment was ceased after 14 weeks when bladders from the males were consistently abnormal. The remaining mice were then maintained on water alone to await tumour development.

Controls. A group of 40 mice (20 males, 20 females), in the same room as the carcinogen-treated mice, were maintained on tap water.

Both control and treated mice were supplied food and water *ad libitum*.

In the hormonally-modified and phenobarbitone-treated mice described below, groups of 6 mice were given either BBN (0·022%) or DBN (0·024%) in the drinking water. Daily consumption of carcinogen was calculated as described above.

Hormonal and metabolic modifications

Castration. Castration was carried out in male and female mice at 3 weeks of age before reaching puberty. Anaesthesia was induced using pentobarbitone 80 mg/kg intraperitoneally (Nembutal Veterinary; Abbott Laboratories) and was maintained with ether.

Males. The abdomen was entered using a small mid-line incision and the immature testes and epididymis excised. In mock castrations the testes were manipulated after laparotomy but were not excised.

Females. Small dorso-lateral incisions were made immediately below each kidney and the ovaries exteriorized. The terminal portion of

each uterine horn was then ligated and the ovary and fallopian tube excised.

In both groups of mice operative and post-operative mortality was zero.

Testosterone was administered in two doses of 25 mg each as a subcutaneous sterile implant (Organon Laboratories) into the interscapular region. The first dose was given at 10 weeks of age and the second 12 weeks later. Phenobarbitone sodium (British Drug Houses) was given as a 1 mg/ml solution in the drinking water. This concentration has been shown to produce a maximum stimulation of liver microsomal activity in rats [9]. Treatment was started one week before carcinogen administration and was continued concurrently until death.

RESULTS

Group A: 0·022% *BBN*

All animals in group A reaching autopsy had malignant tumours of the urinary bladder. No signs of generalized toxicity or primary effects on any other organ were observed. Weight gains of the treated animals were within control values until after the minimum tumour-induction time. Post-mortem examination was precluded in a total of 18 animals (9 males, 9 females) due to extensive cannibalization. These animals have been excluded from the data.

Mice died or became moribund as a result of progressive urinary obstruction with severe hydronephrosis. In many cases where the obstruction was initially unilateral all that remained of the affected kidney was a turgid, urine-filled sac.

As the experiment progressed it became apparent that the males were developing lethal tumors much earlier than the females, indeed a majority had died before palpable nodules had developed in the females. The mean tumour-induction time, calculated as the time elapsing from commencement of treatment until death, occurred 63 days earlier in males than in females, though both groups consumed identical daily doses of carcinogen (Table 1). This is expressed graphically in Fig. 1 in which the cumulative incidence of bladder tumours in males and females is plotted on a probit scale against log time. The parallel lines indicate a normal distribution of induction times and a purely quantitative difference in response. The greater susceptibility of the male mice is not due to an

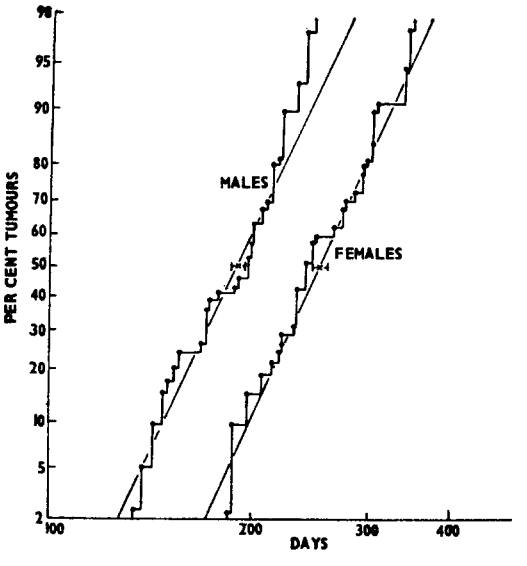

Fig. 1. Cumulative incidence of bladder tumours in male and female C57BL/6 mice treated continuously with BBN (0·022%) in the drinking water. Ordinate: percentage carcinoma incidence, probits. Abscissa: days of treatment, log. Each point represents a day on which one or more mice died. 1—x—1 mean tumour-induction times ± S.E. $P > 0·005$.

Table 1. Bladder tumour-induction times and dosage of carcinogen in male and female C57BL/6 mice treated with BBN (0·022%) continuously in the drinking water.

Sex and No. with tumours (percentage of total)		Daily dose (mg/kg)	Total dose (mg/kg)	Tumour-induction* time (days)
Male				
41	(100%)	35·3 ± 0·6	6724 ± 196	190 ± 4·8†
Female				
41	(100%)	35·9 ± 0·4	9140 ± 335	253 ± 6·9

Mean ± standard error.
*Calculated as the time from starting treatment until death.
†Significantly different ($P > 0·005$) from corresponding females.

enhanced tumour-growth rate in this sex, since the progression from a small just palpable nodule to a large obstructive mass was found to take approximately 30 days in either sex.

Pathology. Tumours induced by BBN were epidermal in origin, of a malignant or highly malignant nature and were classified as anaplastic carcinomas (Fig. 2). A great deal of pleomorphism was apparent. Both glandular and squamous metaplasia was frequently observed (Fig. 3). In all cases, histological examination showed that penetration of the muscle wall of the bladder had occurred giving the bladder a nodular appearance. Adhesions to the surrounding tissues were frequent. In a total of 13 mice (3 males, 10 females) metastases were found in the abdominal lymph nodes which were invaded by cells of a similar morphology to the primary bladder tumour (Fig. 4). In a few cases spread also occurred by peritoneal seeding with numerous metastatic nodules attached to the diaphragm, intestinal mesentery and abdominal wall (Fig. 5). No metastases were observed outside the peritoneal cavity.

Group B

This group received 0·1 % BBN in the drinking water which resulted in a mean consumption of 110 mg/kg/day in males and females. Pathological changes in the bladder were observed in both sexes after only 2 weeks treatment and were characterized by an increase in depth of the stroma underlying the epithelium. Whereas this was the only change seen in the female until 18 weeks after commencement of treatment, the bladder epithelium of the male was damaged at an early stage. Although after 2 weeks of treatment only small areas of ulceration were seen, progressive hyperplasia of the male epithelium developed between 4 and 12 weeks of treatment (Fig. 6). By week 14, when treatment was ended, epithelial downgrowths were observed, which by week 16 had become isolated to form cell nests of von Brunn (Fig. 7). This was the last stage identifiable before the onset of malignancy which was first observed in a male at week 20.

In females the first hyperplastic response was seen after 18 weeks and the first carcinoma 25 weeks after starting treatment. Of the 10 males and 10 females remaining after 20 weeks, 5 males and 2 females developed bladder carcinomas. The others were tumour-free when autopsied at week 40. The mean tumour-induction times for males and females were 22 and 28 weeks respectively. In 5 of these mice with bladder tumours, metastatic spread had occurred within the abdominal cavity. In one of these mice a solitary metastatic focus was found in the lung (Fig. 8).

Controls were all tumour-free at 18 months of age. No reports of spontaneous bladder tumours in this strain have been found.

Effects of hormonal and metabolic modifications on tumour induction times

The sex difference in susceptibility to the carcinogenic action of BBN can be abolished either by castrating males or conversely by treating females with testosterone. Administration of testosterone to castrated males restores the tumour-induction time in these animals to control values. Castration of females did not alter the tumour-induction time (Table 2A and 2B).

Phenobarbitone. No effects on tumour-induction time were detected when phenobarbitone was given concurrently with BBN to normal males and females or to castrated males. In contrast this compound significantly prolonged the induction time of tumours induced by DBN in both sexes and furthermore decreased the number of bladder tumours in males from 5/6, in the DBN-treated controls, to 1/6 in the males additionally treated with phenobarbitone. The already low bladder tumour incidence in females [5] fell from 1/6 to 0/6. Thus, phenobarbitone protects more completely against the carcinogenic effects of DBN on the bladder than on the oesophagus.

A secondary effect of the additional treatment received by these animals was a variation in the consumption of drinking water and a consequent non-uniformity in the dosage of carcinogen. For reasons to be discussed later, these variations are not thought to seriously influence the conclusions.

DISCUSSION

The high degree of carcinogenic potency shown by BBN in the mouse and the rat [3, 10], and the apparent lack of toxic or carcinogenic activity on organs other than the bladder, makes this compound of great potential use as an experimental bladder carcinogen. Its use for this purpose has many advantages over compounds hitherto employed, which if selectively active on the bladder are of low potency [11] or induce a range of tumours at other sites [5, 12, 13]. The only compound other than BBN that has been reported to give rise to a

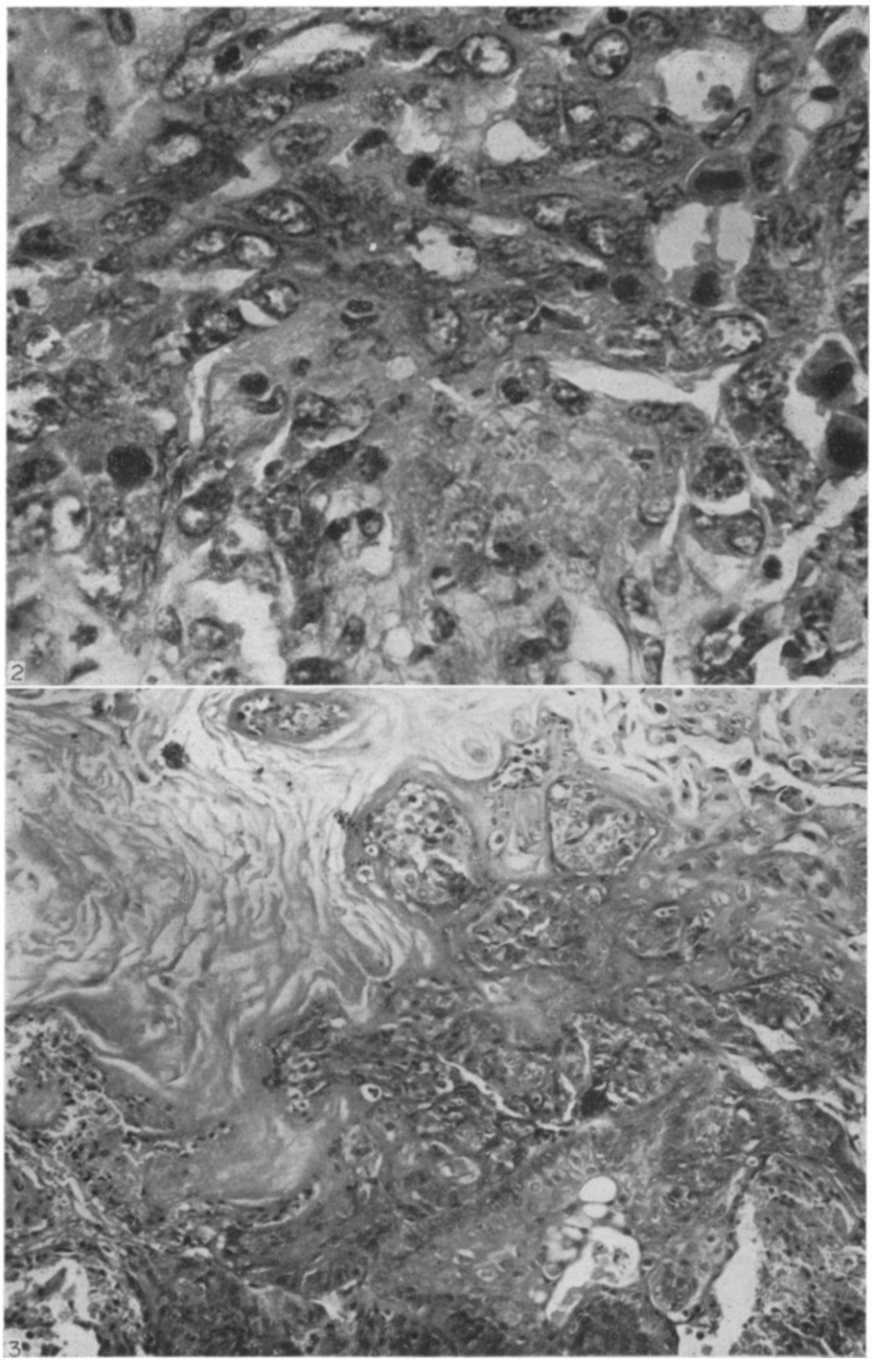

Fig. 2. *Anaplastic bladder carcinoma in a female mouse treated with BBN* (0·022%) *in the drinking water for* 241 *days.* × 1300.

Fig. 3. *Area of squamous metaplasia in the tumour described above. This illustrates the very pleomorphic histological picture observed in most of the tumours examined.* × 200.

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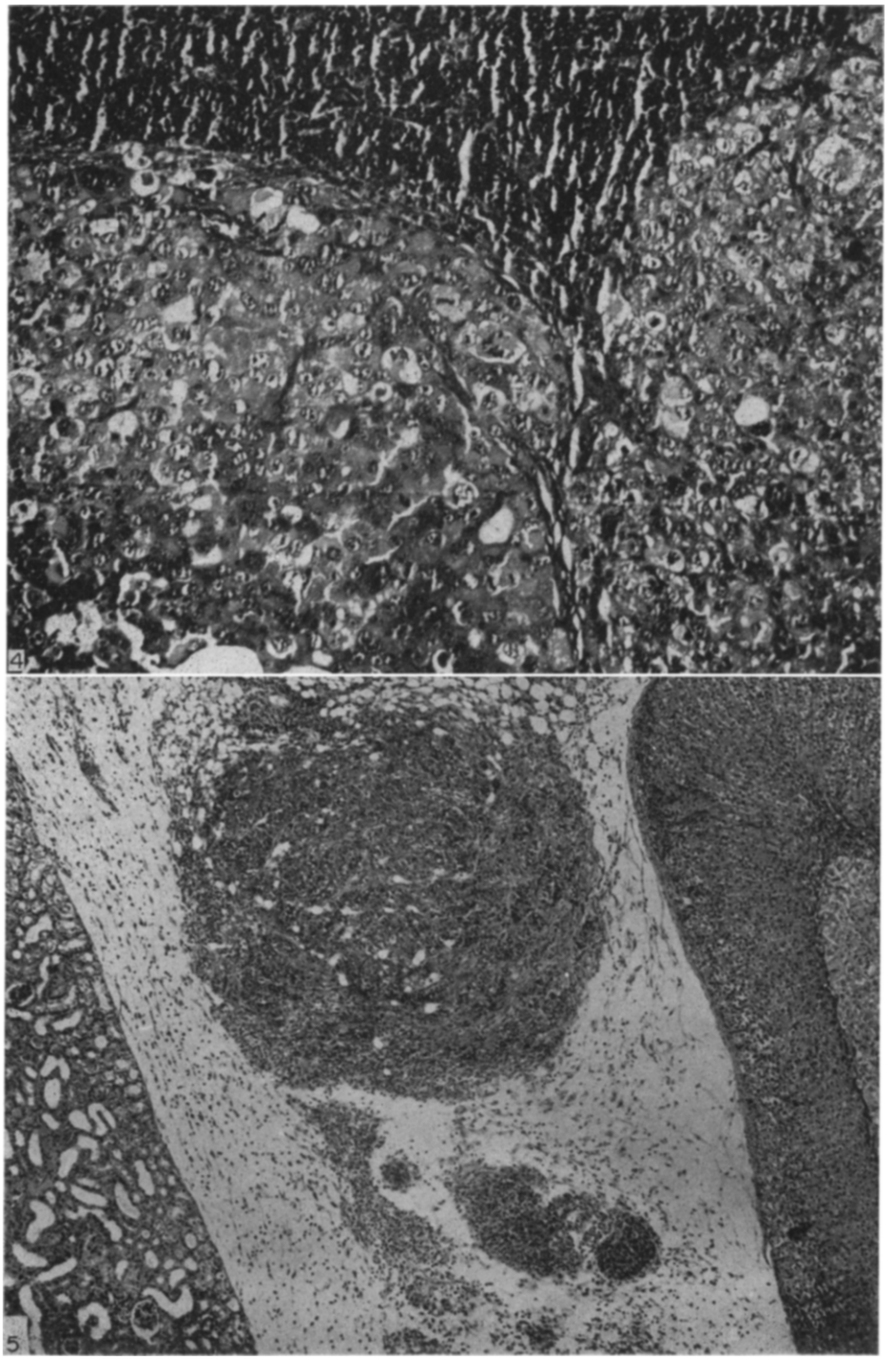

Fig. 4. *Portion of an abdominal lymph node metastasis from the primary bladder carcinoma described in Figs.* 2 *and* 3. *Junction between the lymphoid tissue (top) and the metastasis. Note the marked cellular vacuolization.* × 400.

Fig. 5. *Metastasis from a primary bladder carcinoma lodged in connective tissue and fat between the upper pole of the kidney (left) and the adrenal gland (right). Note the hydronephrosis caused by the primary bladder carcinoma.* × 80.

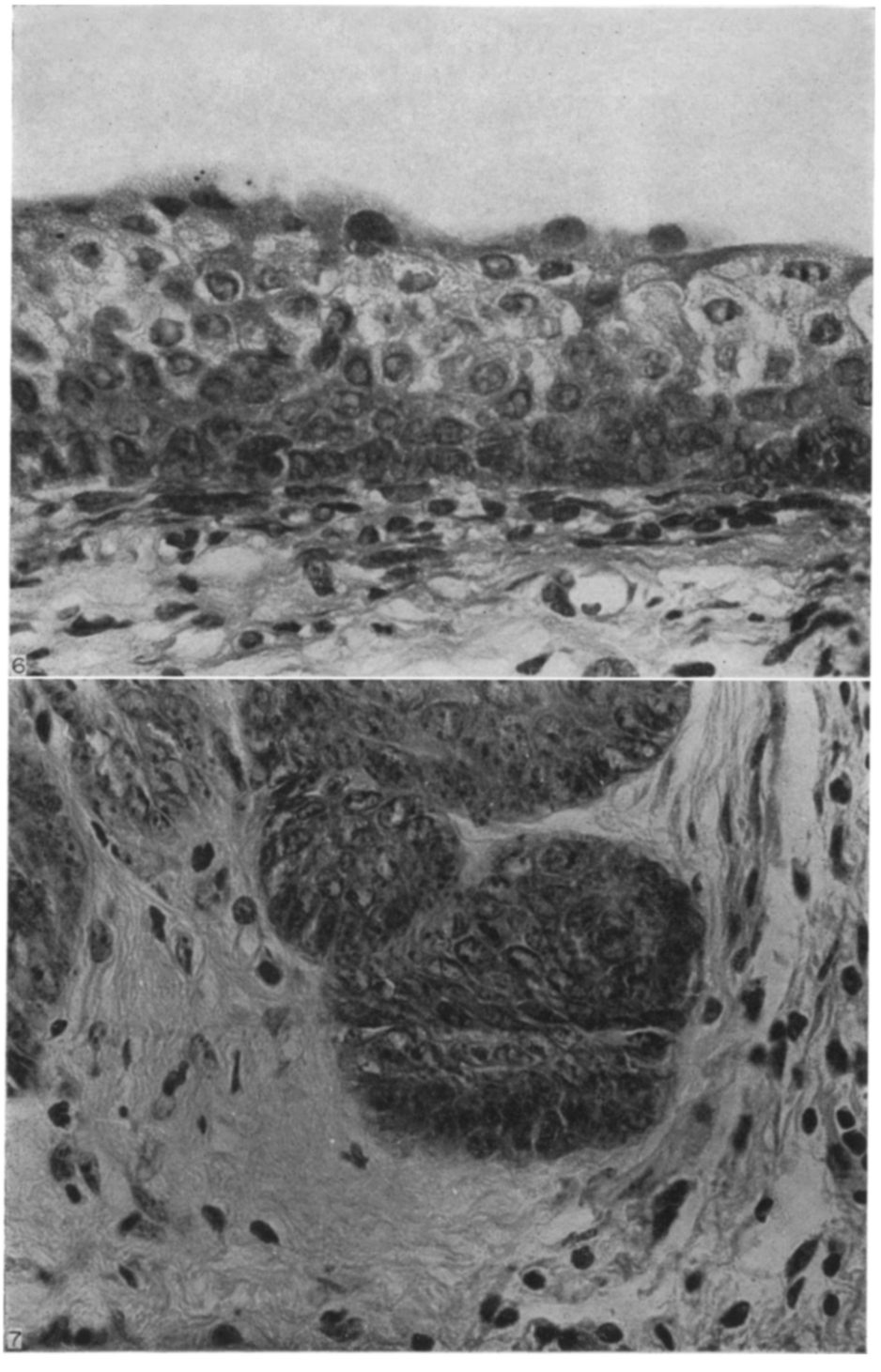

Fig. 6. *Intense hyperplasia of the bladder epithelium of a male after* 12 *weeks teatment with BBN* (0.1%) *in the drinking water. The epithelium is normally* 2 *to* 3 *cells deep.* × 800.

Fig. 7. *Bladder epithelium of a male* 16 *weeks after starting treatment with BBN* (0.1%) *in the drinking water. Epithelial downgrowths have become isolated deep in the stroma between the epithelium* (*left*) *and the smooth muscle* (*right*). *This was the last stage identifiable before the onset of frank carcinoma.* × 800.

Fig. 8. Metastasis to lung from a primary bladder carcinoma in a male mouse treated with BBN (0.1%) for 14 weeks. The animal died of urinary obstruction 8 weeks after treatment was ceased. The metastasis is lodged in the extreme tip of the lobe. × 200.

Table 2A, B. Tumour induction times in male (2A) and female (2B) mice given either BBN 0·022% or DBN 0·024% in the drinking water and subjected to the additional treatments listed. Each group contained 6 mice

Carcinogen	Additional treatment	Tumour-induction* time (days)	Dose of carcinogen* mg/kg/day
Table 2A. Males			
BBN	None	196 ± 15·6	34·4 ± 0·4
BBN	Castration	285 ± 28·7	23·7 ± 0·2
BBN	Sham castration	233 ± 12·9	30·4 ± 0·3
BBN	Castration + testosterone	196 ± 6·8	44·2 ± 0·7
BBN	Phenobarbitone	225 ± 19·3	32·1 ± 0·1
BBN	Phenobarbitone + castration	291 ± 18·9	22·9 ± 0·9
DBN	None	212 ± 10·0	33·9 ± 0·1
DBN	Phenobarbitone	304 ± 8·9	14·4 ± 0·1
Table 2B. Females			
BBN	None	262 ± 22·6	36·6 ± 0·5
BBN	Castration	255 ± 20·8	20·8 ± 0·2
BBN	Testosterone	212 ± 17·1	46·7 ± 1·2
BBN	Phenobarbitone	260 ± 13·9	28·1 ± 3·4
DBN	None	250 ± 8·0	30·5 ± 0·7
DBN	Phenobarbitone	324 ± 12·3	17·6 ± 0·1

*Means ± standard error.

100% incidence of bladder tumours is N-[5-nitro-2-furyl)-2-thiazole] formamide when administered in the diet to rats to a total dose of 16·5 g/rat over 46 weeks [14]. This compares poorly in potency with BBN which requires a mean carcinogenic dose of only 0·2 g/mouse over 29 weeks.

The pathogenesis of bladder cancer as observed in the mice treated with 0·1% BBN (Figs 6–9) corresponds closely to that previously described in mice treated with carcinogens [13] or subject to bladder implantation [15, 16], in rats treated with carcinogens [14] and to many aspects of the disease in humans [17]. It thus provides a valid model for further investigation of the early development and progression of bladder cancer. In addition the high incidence of metastatic spread observed in these animals is unusual for chemically-induced primary tumours and may prove of value in the investigation of this aspect of the disease.

The specificity of action of BBN towards the bladder epithelium is quite remarkable especially in view of the apparent lack of toxicity shown by this compound towards the epithelium of the ureter and renal pelvis. This organ specificity was consistently observed even in mice treated with 0·1% BBN which rapidly induced pathological changes in the bladder epithelium of the male. If the urogenous theory of bladder carcinogenesis is accepted, as has been demonstrated for β-naphthylamine in the dog [18] and ethylsulphonyl-naphthylene-1-sulphonamide in the rat [19], it is difficult to understand why the epithelium of the renal pelvis and ureter should be totally resistant to the carcinogenic action of BBN and also DBN [5]. This resistance occurs not only in the C57BL/6 mouse used in the present study, but also in the Wistar [11] and BD rat [3] and in the case of DBN in the C57 × 1F [20] and ICR mouse [21], the BD rat [3] and the Syrian and Chinese hamsters [22]. These portions of the urothelium are not totally resistant to urinary carcinogens as is shown by the induction of tumours of the renal pelvis in the 1F mouse [13] and the rabbit [23] by 2-acetyl-aminofluorene. Similarly, the bladder carcinogen N-[4-(5-nitro-2-furyl)-2-thiazole] formamide induces severe hyperplasia of the renal pelvis which can progress to carcinoma [14]. Presumably the resistance of these tissues to BBN and DBN must be due either to a lack of tissue-specific activating enzymes [24], or to a requirement for storage within the bladder.

Sex differences in response to bladder carcinogens [14, 25, 26], and to bladder implantation [12], have been previously reported, but quantitation has hitherto been difficult because of the low potency or lack of specificity of

previously available carcinogens. It is of interest that in humans the bladder cancer incidence is consistently higher in males than in females [27]. The results shown in Fig. 1 demonstrate clearly the pronounced sex difference in response, in spite of an identical daily consumption of carcinogen (Table 1). This phenomenon is not due to the slower growth of tumours in females since the onset of the pre-malignant stage was also much delayed in females, furthermore the progression from a small nodule to large obstructive mass occurred at a similar rate in both sexes.

The sex difference in response to BBN is determined by the sex hormonal status of the animal (Tables 2A and 2B). Thus castrated males adopt the female pattern of response and testosterone-treated females adopt the male pattern. Testosterone will restore the response of castrated males to normal. It is unfortunate that the additional treatments listed in Tables 2A and 2B had the effect of altering the daily consumption of carcinogen. From the results reported here with the higher concentration of BBN and from previous results with DBN [5] we believe that the variations in daily dosage between groups are insufficient to explain the observed differences in tumour-induction times. Thus a fourfold increase in the daily dose of DBN decreases the tumour-induction time by only 25 days in males and 12 days in females [5]. The present results with two doses of BBN show similar dose-response characteristics.

The relevant mode of action of testosterone in this system is unknown. It may directly effect the bladder epithelium or bladder physiology, or alternatively have an indirect action on the metabolism and excretion of the carcinogen.

Sex differences have been stated to exist in the level of β-glucuronidase in the urine and bladder [28] and also in the mitotic rate of a variety of epithelial tissues of the mouse, though the urothelium was unfortunately not studied [29]. Both factors have been implicated in bladder carcinogenesis [24, 25]. Although these factors may influence the response to weak carcinogenic stimuli, such as a bladder implant [12], they are unlikely to contribute significantly to the response to a potent stimulus, since merely changing the route of administration of 2-acetylaminofluorene has been found to reverse the sex ratio of bladder tumours in the mouse [13]. Furthermore, the concurrent administration of 1-4-saccharolactone, a specific inhibitor of β-glucuronidase, has failed to influence the carcinogenic response to either β-naphthylamine [24] or BBN [32] in the dog and mouse respectively.

The physiology of the bladder can differ markedly in males and females and urinary retention in the human male is thought to contribute to the increased bladder cancer incidence in this sex [31]. In 60% of male C57BL/6 mice, urinary retention can be demonstrated radiologically and is correlated with the presence of a proteinaceous plug within the urethra. However, this factor can have only minor significance in the present case since testosterone decreased the tumour induction time in females without leading to urinary retention or the production of proteinaceous plugs [32].

In addition to its possible direct effects on the bladder, testosterone has been shown to stimulate the liver microsomal enzyme system of the rat responsible for drug metabolism [33, 34]. It is clear that such sex-linked differences could have profound effects on the response to systemically acting carcinogens, and it may be significant that N-hydroxy-N-2-fluorenylacetamide, which is more carcinogenic to male than female rats, is metabolized differently in the two sexes [35]. It is not clear whether testosterone similarly enhances drug metabolism in the mouse since contradictory reports have appeared in the literature, claiming either no difference in metabolism between the sexes [33] or an effect opposite to that observed in the rat, i.e. female mice metabolize hexobarbitone at a faster rate than males [36]. In agreement with these latter authors we have found that female C57BL/6 mice metabolize [^{14}C] DBN more completely to $^{14}CO_2$, and excrete proportionately less ^{14}C in the urine than do comparable males [32]. The potentiation of the carcinogenic response in females to BBN by testosterone (Tables 2A and 2B) could be due to inhibition of routes of metabolism leading to detoxication or conversely to stimulation of a metabolic pathway leading to production of the proximal urinary carcinogen.

Stimulation of the liver microsomal enzyme system by phenobarbitone failed to influence the induction of bladder tumours by BBN, but significantly protected mice against the induction of bladder and oesophageal tumours by DBN (Tables 2A and 2B). In this system therefore phenobarbitone stimulates detoxication rather than metabolic activation of the nitroso group. The finding that phenobarbitone similarly protects against hepatotoxicity induced by diethylnitrosamine [37], infers that metabolism of the nitrosamines does not necessarily lead to the production of toxic products by the

mechanism proposed by Heath [38]. It can also be inferred that the protective action of phenobarbitone on the induction of oesophageal tumours is due to a reduction in blood level of DBN or a metabolite, since phenobarbitone has not been shown to influence metabolism in organs other than the liver [39]. This implies that DBN may reach the oesophagus indirectly via the blood stream as has been demonstrated in the rat [3].

The similarity between the chemical structure of DBN and BBN, their site of action on the bladder and the existence of common urinary metabolites (in preparation), makes it likely that both are metabolized to the same urinary carcinogen.

In order that future carcinogenic hazards are recognized, it is of great importance that metabolic reactions leading to the activation of carcinogens are fully understood. The chemical simplicity of the agents used here may well aid this understanding. In addition the high potency of BBN provides an extremely useful system for the further investigation of bladder carcinogenesis.

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Transformation of Hamster Cells *in vitro* by 1-β-D Arabinofuranosylcytosine, 5-Fluorodeoxyuridine and Hydroxyurea

P. A. JONES, J. V. TADERERA and A. O. HAWTREY

Department of Biochemistry, University of Rhodesia, P.O. Box MP 167, *Mount Pleasant, Salisbury.*

Abstract—*The transformation of hamster embryo cells by three inhibitors of DNA synthesis, 1-β-D-arabinofuranosylcytosine, 5-fluorodeoxyuridine and hydroxyurea was studied. It was found that transformation was the predictable outcome of a 24-hr exposure to these inhibitors. Dose and time response to 1-β-D-arabinofuranosylcytosine was also investigated and transformation was observed after only 1 hr exposure to the drug. Transformation was verified in all experiments by tumour production, and tumours arose in all animals injected. A correlation between the extent of inhibition of DNA synthesis and transformation appears to exist. It was also shown that the simultaneous addition of a hundred-fold excess of 2'-deoxycytidine was effective in preventing transformation by 1-β-D-arabinofuranosylcytosine. This system has several advantages over others used to investigate chemical carcinogenesis and transformation and cytotoxicity could be separated.*

INTRODUCTION

1-β-D-ARABINOFURANOSYLCYTOSINE which is a potent antileukaemic agent specifically inhibits DNA synthesis in bacterial and animal cells [1, 2]. The mechanism of action of the drug has been investigated in a variety of animal cells [3–7]. These include HeLA cells [3], Don-C hamster fibroblasts [4, 5] and mouse L-cells [6, 7]. Since the tissue culture cells mentioned are all line cells it appeared of interest to investigate the effect of the drug on normal cells. We have used the hamster embryo cell system to investigate the possibility of transformation by the drug. This system has also been used to investigate aromatic hydrocarbon carcinogenesis [8–10]. Results of these experiments are reported here and it is shown that 1-β-D-arabinofuranosylcytosine is a transforming agent in the hamster embryo system. Experiments with two other inhibitors of DNA synthesis, 5-fluorodeoxyuridine and hydroxyurea demonstrated that they were also effective in inducing transformation in this system. Attempts were also made to determine the minimum transforming dose and minimum treatment time, and to correlate the extent of DNA synthesis inhibition with the transformation observed.

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*The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; dC, 2'-deoxycytidine; HU, hydroxyurea; FUdR, 5-fluorodeoxyuridine.

MATERIAL AND METHODS

Chemicals

The hydrochlorides of ara-C* and dC were obtained from the Sigma Chemical Company, St. Louis, Missouri, HU was purchased from Mann Research Laboratories, New York. FUdR was a gift from F. Hoffman La Roche, Basle, Switzerland. ^{3}H-methyl thymidine (specific activity 17·9 C/m-mole) was obtained from Schwarz/Mann, Orangeburg, New York. The sterile aqueous solution supplied was diluted with whole medium to give a final activity of 1 μC/ml.

Cell cultures

Primary cultures of hamster embryo cells were prepared by the trypsinization of decapitated and degutted 12- to 13-day old embryos. Cells were grown in 4-oz prescription bottles using Eagle's Minimal Essential Medium containing Earle's Salts (Schwarz/Mann, Orangeburg, New York). The basal medium was supplemented with 10% calf serum prepared from pooled calf blood, 10% tryptose phosphate broth (Oxoid Ltd., London, S.E.1, England) and penicillin and streptomycin (CAPS. Ltd., Salisbury, Rhodesia). Cultures were incubated at 37°C and examined daily on an inverted microscope.

Treatment of cultures

The treatment of cells with the test compounds was standardized. Monolayer primary cultures were trypsinised and secondary cultures seeded in 4-oz prescription bottles at a concentration of 2×10^6 cells per bottle. Treatment of cultures was always started 24 hr after the cells had been replated as secondary cultures. Solutions of test compounds were made up in basal medium, filter sterilized and then diluted with fully constituted medium to give the required concentration. After the required treatment time, test medium was replaced with normal medium. Medium in cultures exposed to ara-C was changed for medium containing 10^{-3}M dC for a further 24 hr.

Measurement of thymidine incorporation

The relative incorporations of ^{3}H-methyl thymidine by treated and untreated cultures was used to estimate the inhibition of DNA synthesis. The medium in duplicate bottles of treated and untreated cultures was changed to medium containing ^{3}H-methyl thymidine and still containing test compound in the case of treated cultures. After 2 hr the cells were harvested, washed twice in phosphate-buffered saline and then suspended in phosphate-buffered saline. Cell number was estimated in a haemacytometer and radioactive uptake was determined by precipitation with cold trichloroacetic acid followed by Millipore filtration. The acid insoluble material was counted in toluene containing 0·5% (w/v) 2,5-diphenyloxazole and 0·03% (w/v) 1,4-bis-(5-phenyloxazole-2-yl) benzene in a Packard Scintillation Spectrometer Model 2002.

Inoculation of cells into hamsters

Cells to be tested for tumour-forming ability were harvested with trypsin and suspended in basal medium at a concentration of 2×10^7 cells per ml. They were then injected subcutaneously into young hamsters, each animal receiving an inoculum of 0·5 ml containing 1×10^7 cells. Animals were examined regularly for the appearance of tumours.

RESULTS

In the first series of experiments secondary cultures were treated with 10^{-3}M ara-C for 24 hr and then with 10^{-3}M dC for a further 24 hr. This treatment appeared to withhold cells from division. Toxicity as evidenced by cell death, and the appearance of giant cells and an increased length of time required to monolayer, became apparent. No such toxicity was seen in control cultures which were treated with 10^{-3}M dC for 24 hr. Areas of active growth of the surviving cells became apparent approximately 14 days after the initial exposure (range 9 to 24 days) and clones of fibroblastic fusiform cells became visible against a background of dying cells. These clones were easily visible to the naked eye. With subsequent passages these fusiform cells dominated the culture and exhibited a random growth pattern, criss-crossing, multilayered growth and the ability to grow in culture indefinitely. Cells treated with 10^{-3}M dC for 24 hr on the other hand did not show the development of clones and could only be maintained in culture for about 30 days (± 8 passages) after which they deteriorated rapidly and were discarded. Cells exposed to 10^{-3}M ara-C did not recover if the subsequent treatment with 10^{-3}M dC was omitted.

In our experiments the following criteria were used to define the transformed state: (a) the ability of cells to grow in culture for an indefinite period as compared with the limited life-span of controls; (b) the fusiform shape of the cells coupled with a random, criss-crossed growth pattern; and (c) the ability to produce tumours when injected into young hamsters. To assess the reproducibility of the system four separate experiments were conducted by exposing cells to 10^{-3}M ara-C for 24 hr. In all of these experiments transformation was observed.

The tumour-forming abilities of cells from each experiment are summarized in Table 1. The time taken for tumours to develop is related to the length of time in culture as seen in Experiment 1. Cells from Experiment 1 have been maintained in culture continuously for 13 months and have at present been passaged 100 times. Control cells failed to produce

Table 1. *The development of tumours in hamsters injected with cells transformed* in vitro *via treatment of cultures with* $10^{-3}M$ *ara-C for* 24 *hr.*

Experiment	Passage in which cells were injected	No. of tumours/ No. of animals injected	No. of instances metastases observed/ No. of animals injected	Development time (weeks)
1	5th	10/10	10/10	5
	10th	10/10	10/10	3
	14th	10/10	0/10	2
2	12th	9/9	0/9	3
3	9th	9/9	0/9	5
4	12th	10/10	10/10	4

tumours when injected under the same conditions. The primary tumours which arose at the site of injection were classified as malignant fibrosarcomas and metastasis was observed in some experiments (Table 1). Secondary tumours were very widespread and in general it was found that animals with secondary tumours had small primary tumours.

Table 2 shows experiments conducted in an attempt to find the minimum transforming dose of ara-C. Transformation was observed at a concentration of 10^{-6}M but not at 10^{-7}M ara-C. Toxicity was assigned arbitrary values of high, medium and low by visual assessment as described earlier. Thus no correlation between gross toxicity and transformation is apparent.

Cultures were also exposed to 10^{-3}M ara-C for varying times as described in Table 3. Transformation was observed at all the treatment times tried, and it was again apparent that there was no correlation between toxicity and transformation.

The extent of the inhibition of DNA synthesis by varying concentrations of ara-C was studied in the experiments reported in Table 4. The inhibition of DNA synthesis was measured at two different time intervals after initial exposure and an attempt made to correlate the size of the dose with transforming ability. Thus 10^{-6}M ara-C inhibits synthesis by 62% after 24 hr and induces transformation whereas a dose of 10^{-7}M only inhibits synthesis by 35% and does not transform. Protection against transformation was afforded by the simultaneous addition of 10^{-3}M dC to a test solution of 10^{-5}M ara-C and the level of inhibition is again low at 34% after 24 hr. Equal concentrations of ara-C and dC (10^{-3}M) were ineffective in preventing inhibition of synthesis and transformation occurred in this case.

Table 2. *Transformation of hamster embryo cells by different concentrations of ara-C. Cells were treated with the indicated concentrations of ara-C for* 24 *hr and then with* $10^{-3}M$ *dC for a further* 24 *hr. Transformation was verified by tumour production as in Table* 1 *and values for toxicity assessed visually.*

Concentration of ara-C (M)	Transformation	Toxicity
10^{-3}	Yes	High
10^{-4}	Yes	Medium
10^{-5}	Yes	Medium
10^{-6}	Yes	Low
10^{-7}	No	Low

Table 3. *The time required for transformation by* 10^{-3}M *ara-C. Cells were exposed to* $10^{-3}M$ *ara-C for the indicated times and then to* $10^{-3}M$ *dC for a further* 24 *hr. Transformation and toxicity were assessed as in Table* 2.

Duration of treatment at 10^{-3}M ara-C (hr)	Transformation	Toxicity
1	Yes	Low
2	Yes	Low
4	Yes	Medium
8	Yes	Medium
24	Yes	High

To investigate the possibility that other inhibitors of DNA synthesis were transforming agents in the hamster embryo system, cultures were exposed to 10^{-6}M FUdR and 10^{-2}M HU for 24 hr. Medium was then changed for fresh medium and clones were observed 12 days after treatment. Transformation was verified by tumour production.

DISCUSSION

The cytotoxicity and the production of chromosome breaks by ara-C has been demonstrated [4–7, 11], and breaks in the DNA of

Table 4. The inhibition of DNA synthesis by different concentrations of ara-C and reversal of this inhibition by simultaneous treatment with dC. Cells were treated as indicated and the inhibition of DNA synthesis as compared with untreated cultures determined 5 and 24 hr after treatment as described in Material and Methods.

Treatment	Inhibition of DNA synthesis after 5 hr (%)	Inhibition of DNA synthesis after 24 hr (%)	Transformation
10^{-3}M ara-C	92	88	Yes
10^{-5}M ara-C	96	82	Yes
10^{-6}M ara-C	68	62	Yes
10^{-7}M ara-C	29	35	No
10^{-5}M ara-C with 10^{-3}M dC	28	34	No
10^{-3} ara-C with 10^{-3}M dC	81	71	Yes

cells treated with HU have been reported [12]. By using normal hamster embryo cells we have shown that transformation is the predictable outcome of exposure of cultures to certain inhibitors of DNA synthesis. The possibility therefore exists that certain agents of chromosome breakage in this system are potentially carcinogenic. Recently, however, Benedict *et al.* [13] have correlated aneuploidy with transformation, whereas chromosome breaks were related to cytotoxicity in hamster embryo cells treated with polycyclic hydrocarbons. Therefore aneuploidy rather than chromosome breakage may be more important in ara-C induced transformation. The biological sites of action of the three inhibitors used are known, ara-C is thought to act at the level of the polymerization reaction [14], whereas FUdR specifically inhibits thymidilate synthetase [15] and HU interferes with the ribonucleoside diphosphate reductase reaction [16–18], and their use as transforming agents in the hamster embryo system should prove useful in studying chemical carcinogenesis.

Our experiments to determine dose and time response to ara-C in mass culture are of necessity crude, and cloning experiments to measure percentage transformation such as those described by Berwald and Sachs [8] would yield more accurate results. The results do show, however, that transformation can occur without the gross toxicity that usually accompanies exposure of cultures to chemical carcinogens. Similarly, the attempted correlation between the inhibition of DNA synthesis and transformation would be better shown by cloning experiments. Nevertheless it appears possible that a correlation does exist between the extent of inhibition of DNA synthesis and transformation. It is also of interest that transformation can be prevented by the simultaneous addition of a hundred-fold excess of dC. The simultaneous addition of dC has been shown to prevent chromosome breakage in Don-C cells [5].

The inhibitors used in this study are cell cycle-specific agents and thus a definite physiological state of the cell may be required for transformation. Benedict *et al.* [5] have shown that maximum sensitivity to ara-C occurs during the latter half of S and the beginning half of G_2. Experiments are currently under way to determine whether transformation is also cell cycle-specific.

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A- and C-Type Particles of Mouse Myeloma Cells

A. KARPAS and J. CAWLEY

Department of Medicine, University of Cambridge, England.

Abstract—*Electron microscopic examination of the P-3 mouse myeloma cell line revealed that most cells contained a very large number of intracisternal A-type particles, while only a few C-type particles appeared to be produced. The C-type particle was readily isolated in 3T3 mouse fibroblast cells where its presence could be demonstrated both by the RIF test and by electron microscopy, which revealed numerous extracellular C-type particles, but no A-type particles. Since the P-3 cells in contrast contained many more A-type than C-type particles, it is suggested that* (1) *intracisternal A-type particles are not precursors of C-type particles,* (2) *intracisternal A-type particles are not infectious.*

INTRODUCTION

A-TYPE particles are a heterogeneous assortment of intracellular ring-shaped particles measuring approximately 70 mμ in diameter. In the original characterization of A-type particles in mouse mammary carcinoma cells [1], these structures were found in the cytoplasmic matrix and were seen to consist of two clearly defined concentric shells surrounding an electron lucent core.

However, the term A-type particles has also been used to describe a somewhat different type of particle which is found within cisternae of the rough endoplasmic reticulum. Like Bernhard's A-type particles these structures have an electron lucent core but this is smaller in diameter than in Bernhard's particles. Precursor forms of the intracisternal A-particles can be seen budding into cisternae from the membrane of the rough endoplasmic reticulum. Recently, such intracisternal particles have been reported to be present in a range of normal mouse tissues [2]. The relation of both varieties of A-type particles to malignancy is not known, but the role of the C-type virus particle in the induction of malignancy is well documented. The C-type particle measures approximately 100 mμ in diameter and consists of an electron-dense core surrounded by two envelopes (internal and external). C-type particles are found mainly extracellularly or budding from the cell membrane.

While the biological activity of the C-type particle can be readily demonstrated *in vivo* and *in vitro*, all attempts to demonstrate biological activity ascribable to the A-type particle have failed [3, 4].

We have recently reported the first biological isolation of a C-type virus from a protein-producing mouse myeloma cell line (P-3) which carried both intracisternal A- and C-type particles [5]. Earlier, Watson *et al.* [6] had shown that C-type virus particles replicated in several mouse myeloma cell lines, but had failed to demonstrate *in vivo* and *in vitro* biological activity of the virus. In this report we deal only with the intracisternal A-type particles of mouse myeloma cells and present evidence in support of the notion that intracisternal A- and C-type particles are unrelated despite the fact that both may be seen in the same cell.

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MATERIAL AND METHODS

Cells and culture media

P-3 cell line. These cells were a cloned plasma cell line originated by Drs. K. Horitoba and M. Cohn from an oil-induced myeloma of Balb/c mice. The cells were kindly provided by

Dr. C. Milstein who grew them in Dulbecco's modified Eagle's medium (DMM) supplemented with 10% horse serum.

3T3 cell line. This cell line was derived from mouse embryos of the NIH strain, and was obtained from Dr. J. Harvey.

Mouse embryo fibroblasts (MEF). These cells were newly established from foetal Balb/c mice.

Both the 3T3 and MEF cells were grown in DMM supplemented with 10% foetal bovine serum.

Isolation of the C-type virus from P-3 cells

P-3 cells were co-cultivated with 3T3 cells, and tissue culture fluid from the mixed cell culture was passed through a 0·45 mμ. millipore filter, and the filtrate was used for inoculation of normal 3T3 cells. The infected 3T3 cells were trypsinized and subcultured at 10-day intervals. After the fourth trypsinization cells were seeded into 60-mm Falcon plastic plates at a concentration of 5×10^5 cells per plate; a similar number of non-infected 3T3 cells were also seeded on plates. In order to learn whether infected 3T3 cells had become resistant to super-infection with MSV, the resistance-inducing factor (RIF) test was performed [7]. In this test both inoculated and non-inoculated 3T3 cells were infected with murine sarcoma virus, Harvey strain (MSV-H). After 1 hr's incubation at 37°C in a CO_2 incubator, DMM + 5% calf serum was added to each plate. The medium was changed every 2 days and the plates were examined daily for the development of foci of cell transformation.

Preparation of cells for electron microscope examination

P-3 cells, inoculated 3T3 cells, non-inoculated 3T3 cells, fourth passage non-infected MEF cells and MSV-H infected MEF cells were examined. Each of these cell types was separated from culture medium by low speed centrifugation and then fixed as a suspension for 1 hr at room temperature in 1·5% glutaraldehyde on 0·1 M cacodylate-HCl buffer containing 1% sucrose (pH 7·4). The cells were washed three times in 0·1 M cacodylate-HCl buffer containing 7% sucrose (pH 7·4) and post-fixed for 2 hr at room temperature in 2% osmium tetroxide in 0·1 M cacodylate-HCl (pH 7·4). They were then dehydrated in one change each of 70% and 90% ethanol, four changes of absolute ethanol each of 5 min duration, followed by two 15 min changes of propylene oxide. Finally, the cells were embedded in Taab embedding resin. Thin sections were cut with glass knives on an LKB ultratome III, doubly stained with uranyl acetate and lead citrate, and examined in an AEI EM 6B electron microscope operated at 60 Kv

Infection of MEF cells with MSV-H

Since the MEF cells were seen by electron microscopy to contain intracisternal A-type particles, it seemed interesting in view of the possibility that A-type particles might be precursors of C-type virus particles to see if the MEF cells were susceptible to infection and transformation by C-type MSV-H.

To do this, MEF cells were seeded into 60 mm Falcon plastic plates at a concentration of 5×10^5 cells per plate. On the following day, the plates were treated for 30 min with DEAE-Dextran [8], and inoculated with MSV-H. Both infected and non-infected control cells were kept for two weeks and observed daily for cellular transformation. At the end of this period samples of both cultures were processed for electron microscopy.

RESULTS

When examined by electron microscopy almost every P-3 cell was seen to contain numerous A-type particles. These particles were confined to the interior of cisternae of rough endoplasmic reticulum. Although they could be found throughout the cytoplasm, they appeared frequently to be grouped in clusters (Fig. 1). No extracellular A-type particles were seen in any of the cells examined. Occasional extracellular C-type particles were seen (Fig. 2). Normal 3T3 cells did not contain either A- or C-type particles.

Examination of infected 3T3 cells revealed a very large number of mature C-type viral particles outside the cell membrane (Fig. 3), and this was in sharp contrast to the small number of C-type particles in P-3 cells. Occasionally, particles could be seen budding from the 3T3 cell membrane. However, none of the 3T3 cells examined revealed any A-type particles in their cytoplasm.

Biological assay for the presence of C-type particles, using the RIF test, indicated that the 3T3 cells inoculated with filtered culture fluid derived from the 3T3—P-3 co-culture, were resistant to infection and transformation by MSV-H. This was in contrast to the non-infected control 3T3 cells which did show foci of transformation after inoculation of MSV-H.

The absence of A-type particles in the infected 3T3 cells which produced C-type virus indicated that the A-type particles did

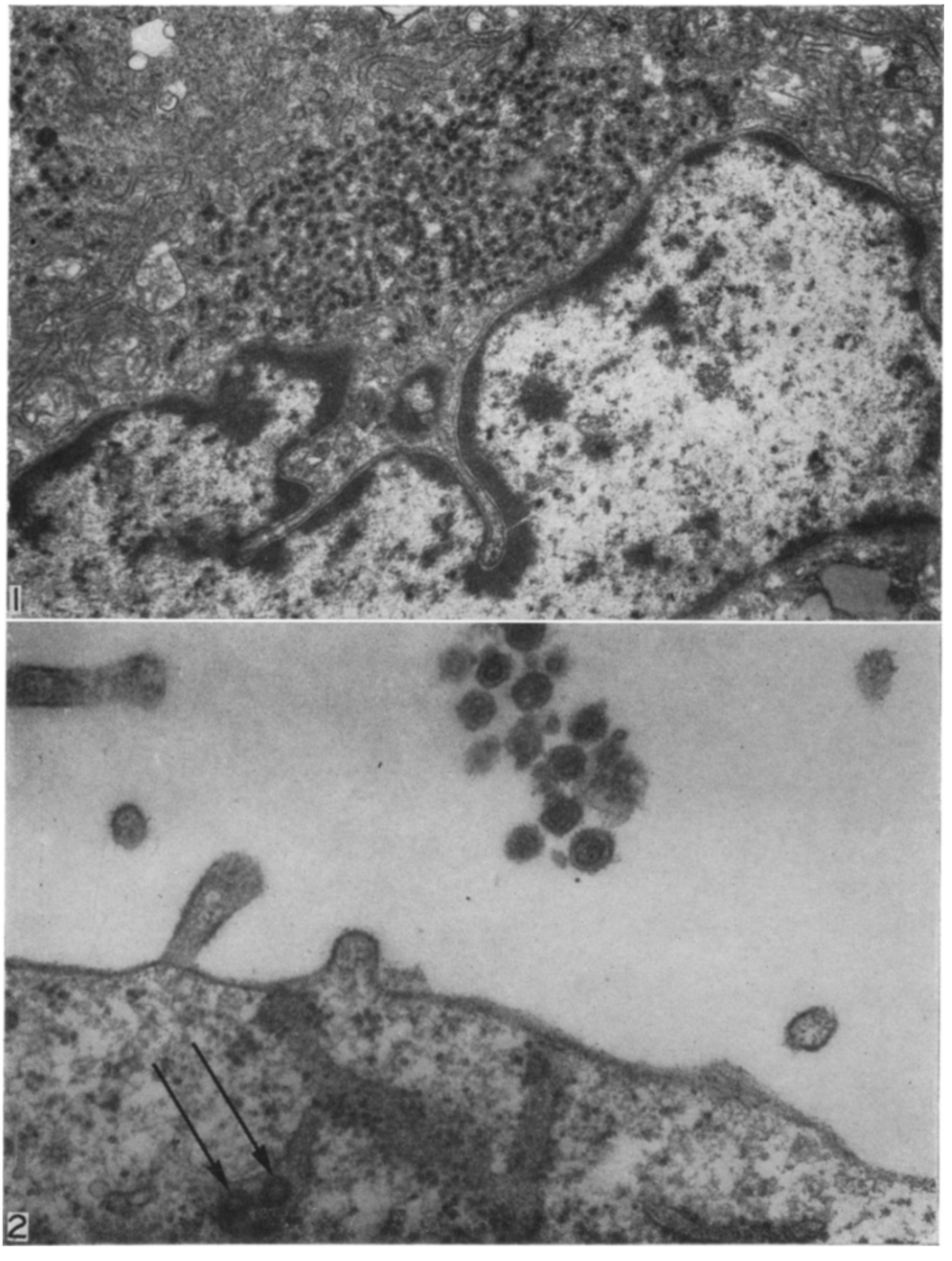

Fig. 1. *P-3 cells showing a cluster of intra-cisternal A-type particles* (× 12,000).

Fig. 2. *P-3 cells containing intra-cisternal A-type particles* (↑) *in the cytoplasm and C-type particles outside the cell membrane,* (× 60,000).

(to face p. 602)

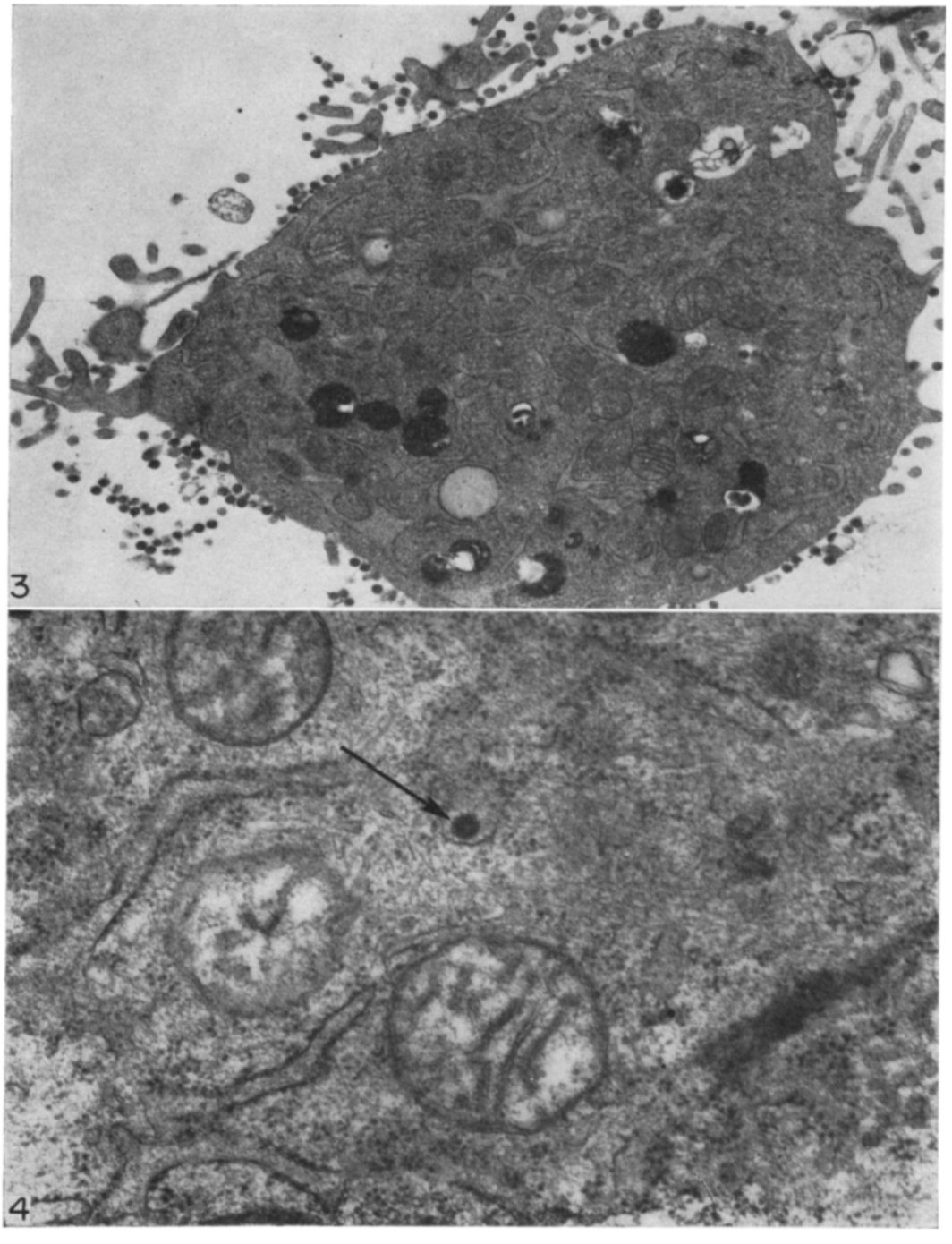

Fig. 3. *A section through the cytoplasm of an infected 3T3 cell showing numerous extracellular C-type particles, but no intra-cisternal A-type particles* (× 12,000).

Fig. 4. *An intra-cisternal A-type particle* (↑) *in normal mouse Balb/c cell culture* (*MEF cells*) (× 48,000).

not infect the 3T3 cells, and were unlikely to be a precursor form of the C-type virus.

Ultrastructural examination of the normal mouse embryo fibroblasts derived from the Balb/c mouse strain revealed that occasional cells contained intracisternal A-type particles (Fig. 4). However, these cells were found to be susceptible to infection and transformation by MSV-H, thus indicating that no interference existed between the A-particles in the cytoplasm and the added C-type virus (MSV-H). As expected the infected MEF cells were seen to produce numerous C-type particles. No increase in the low frequency of intracisternal A-type particles was noted in MSV-H infected MEF cells. Moreover, MEF cells containing both intracisternal A-type particles and budding C-type particles could occasionally be found.

DISCUSSION

Bernhard [1] pioneered the systematic electron microscopic study of tumour cells and their viruses. On the basis of this work he proposed a nomenclature which is still in use. He also proposed that the intracytoplasmic A-type particle might be a precursor of the B-type virus which in the mouse is Bittner's mammary carcinoma virus. Lasfargues *et al.* [9] on the other hand, considered the A-type particle to be a non-specific cell reaction. All attempts to demonstrate any biological activity associated with the intracisternal A-type particle have been unsuccessful [3, 4], although Kerckaert *et al.* [10] have recently reported that partially purified intracytoplasmic A-particles may have biological activity *in vivo*.

Recent studies on the proteins of the inner shell of the A-type particle have suggested differences between their solubility and that o the proteins of murine RNA oncogenic viruses [11].

Wivel and Smith [2] reported that preliminary immunological studies have failed to demonstrate any cross-reactivity between the A-type particle and the murine leukaemia virus and murine mammary tumour virus. These authors reported the presence of such A-type particles in a variety of normal mouse cells. We also found such intra-cisternal particles in normal mouse embryo fibroblasts derived from Balb/c mice. Kuff *et al.* [12] have recently presented data indicating that antiserum to intracisternal A-particles does not react with samples of leukaemia and mammary tumour viruses and conversely that isolated A-particles do not react by complement fixation or immunodiffusion with anti-serum against leukaemia virus antigens.

De Harven and Friend [13] who studied tissues of leukaemic mice, reported the simultaneous presence of A- and C-type particles in the same cells of infiltrated organs. On the basis of these and similar observations, Bernhard [1] suggested that the A-type particle might be a precursor of C-type particles. While almost all the P-3 cells which we used as the source for the isolation of the C-type particles contained A-type particles, only a few contained both A- and C-type particles. While the C-type virus readily infected the 3T3 cells, no A-type particles could be seen in any of these infected 3T3 cells.

Therefore, the evidence derived from the present study supports the notion that A-type particles are probably not related to the C-type virus. The absence of A-type particles in any of the infected 3T3 cells probably suggests that these particles are not infectious. The observation that normal MEF cells of Balb/c strain mice which contained A-type particles, were susceptible to infection and transformation by MSV-H indicates that the presence of A-type particles in these cells does not interfere with infection by a C-type virus. This observation further supports the idea that A-type particles are not precursors of C-type virus. Since A- and C-type particles are evidently unrelated in mouse myeloma cells it is not surprising also to find both particles occurring simultaneously in the same mouse leukaemia cells [14].

However, the basic nature of the intracisternal A-type particles and their relation to malignancy remain unknown.

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The Influence of Azathioprine on Tumorogenesis by 7-12 Dimethylbenz [*a*] Anthracene in Rats

MARCEL DARGENT†, JEAN-JACQUES BOURGOIN, PATRICK NOEL and RICHARD WEISSBROD

Centre Léon Bérard, 28, rue Laënnec, 69 Lyon 8e, France

Abstract—*Azathioprine modifies the carcinogenic effect of 7–12 DMBA: if azathioprine is given regularly from the date of carcinogenic stimulus by DMBA or before this stimulus, tumours appear later, are less numerous, and less frequently malignant than in controls given DMBA alone.*

When azathioprine is only given from the date of appearance of the first tumour, there is a more rapid spread of tumours, but the final number and their malignancy are about the same as if the rats had not been given azathioprine.

THIS study arises from preliminary investigations [1] on the influence of surgery or immunosuppressive agents to modify the number of metastases in a system of DMBA-induced tumours in rats.

MATERIAL AND METHODS

Azathioprine (Imuran, Wellcome Lab.) has been given by a stomach tube, twice a week, throughout the experiment. The dose per rat was 0·05 mg in 1 ml sesame oil as we first established that this dose was the minimum to induce an immuno-suppression to prolong the survival of tail skin grafts between normal rats (Sprague–Dawley × Wistar), longer than 20 days.

7–12 *Dimethylbenz* [a] *anthracene* (9–10 dimethylbenzanthracene, Schuschardt Lab.) has been given by stomach tube in a single dose of 15 mg in 1 ml sesame oil, as we previously established that this dose was the minimum to induce tumours in 100% of rats [1].

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Address for reprints: Dr. Bourgoin, Service d'Immunologie, Centre L. Bérard, 28 rue Laënnec, Lyon 8e.

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Animals. 120 female rats of Sprague–Dawley inbred strain, two months old, weighing 200 g, were divided randomly according to permutation tables into 6 groups of 20, and assigned identification numbers as follows:

Group I first received treatment with azathioprine. Ten days after the beginning, they received the DMBA.

Group II received DMBA alone.

Group III received DMBA, and, from the time of the ingestion of the carcinogenic compound, treatment with azathioprine.

Group IV received DMBA alone, then, when the first tumour appeared (for each animal) received treatment with azathioprine.

Group V received the treatment with azathioprine alone.

Group VI was untreated.

These two last groups are controls, in as much as they did not receive any treatment with DMBA. Group II is the positive DMBA control group. Figure 1 illustrates the experimental design for the four main groups.

All animals were examined twice a week for the experimental period, and the onset and evolution of the tumours were recorded. The

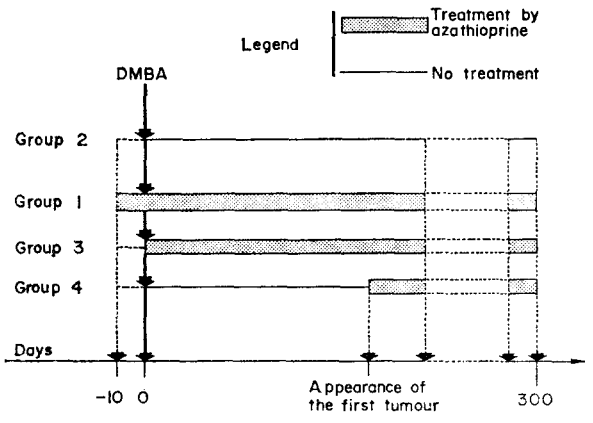

Fig. 1

experiment was stopped at the 300th day as we had previously shown that more than 50% of rats were dead at about this time.

RESULTS

Of the control animals two in group V and three in group VI had palpable tumours when they died. It can be seen, in Table 1, that rats in groups I and III developed fewer tumours per animal than the positive controls, whereas the animals in group IV developed the highest number.

Table 1. *Number of palpable mammary tumours*

	Average number $\pm S/\sqrt{n}$	Limit numbers
Group I	3·2 ± 0.4	1–9
Group II	5·3 ± 0·5	2–11
Group III	3·6 ± 0·5	1–10
Group IV	5·8 ± 0·6	2–13

In groups II and IV, every animal had developed a palpable tumour by day 80, whereas for groups I and III, several tumours appeared later. If the average date of appearance of the first tumour is calculated, there is no significant difference between the second and fourth groups, but there is a definite delay in the appearance of the first tumour in the first and third groups.

The date of development of the second and subsequent tumours is shown in Table 2; the delay for the appearance is marked in groups I and III: there is no significant difference between these groups. Group IV, however, showed an accelerated tumorogenesis after the fourth tumour.

Only 287 of the tumours were examined histologically as, in several cases, separate tumour masses had fused; in 10 cases, tumours were necrotic and unsuitable for histological examination. Fifteen rats out of 19 have had an histological examination in group I (2 rats had no tumour when they were killed at day 300, 2 rats had necrotic tumours). For all the other groups, 20 rats bore mammary tumours. The control groups V and VI provided 5 tumours.

The tumours were divided into three major groups as described previously:

malignant tumours (MT), subdivided into adeno-carcinomas, and undifferentiated carcinomas (Figs. 2 and 3); *benign tumours* (BT) subdivided into adenomas, fibromas and fibro-adenomas (Fig. 4); *histologically ambiguous tumours* (HAT) when it was uncertain if the tumours were benign or malignant (Fig. 5).

The difference between each group in the average number of histologically controlled tumours per rat is given in Table 4. If the number of benign tumours is considered, the average number is about the same for all groups. There is little difference between groups II and IV in the ratio of MT to total number of tumours, but there is some difference between these two groups if the ratio of BT or HAT to total number is considered. Groups I and III however, do show a difference in that there is a considerably higher relative incidence of benign tumours.

Table 5 shows that whereas the majority of animals in groups I, II and IV bore both malignant and benign tumours, the majority of animals

Table 2. *Average date of appearance for the tumours in each group* ($\pm S/\sqrt{n}$)

	Tumour number						
Groups	1st	2nd	3rd	4th	5th	6th	7th
Group I	197 ± 15·0	215 ± 18·0	247 ± 19·0	*	*	*	*
Group II	75 ± 8·0	111 ± 11·2	132 ± 14·0	164 ± 14·0	173 ± 14·0	181 ± 15	190 ± 17
Group III	135 ± 17	179 ± 15	202 ± 18	206 ± 18	217 ± 18	229 ± 18	230 ± 18
Group IV	82 ± 12	128 ± 17	146 ± 19	156 ± 16	160 ± 16	161 ± 16	162 ± 16

*Very few rats in the first group had more than 3 tumours.

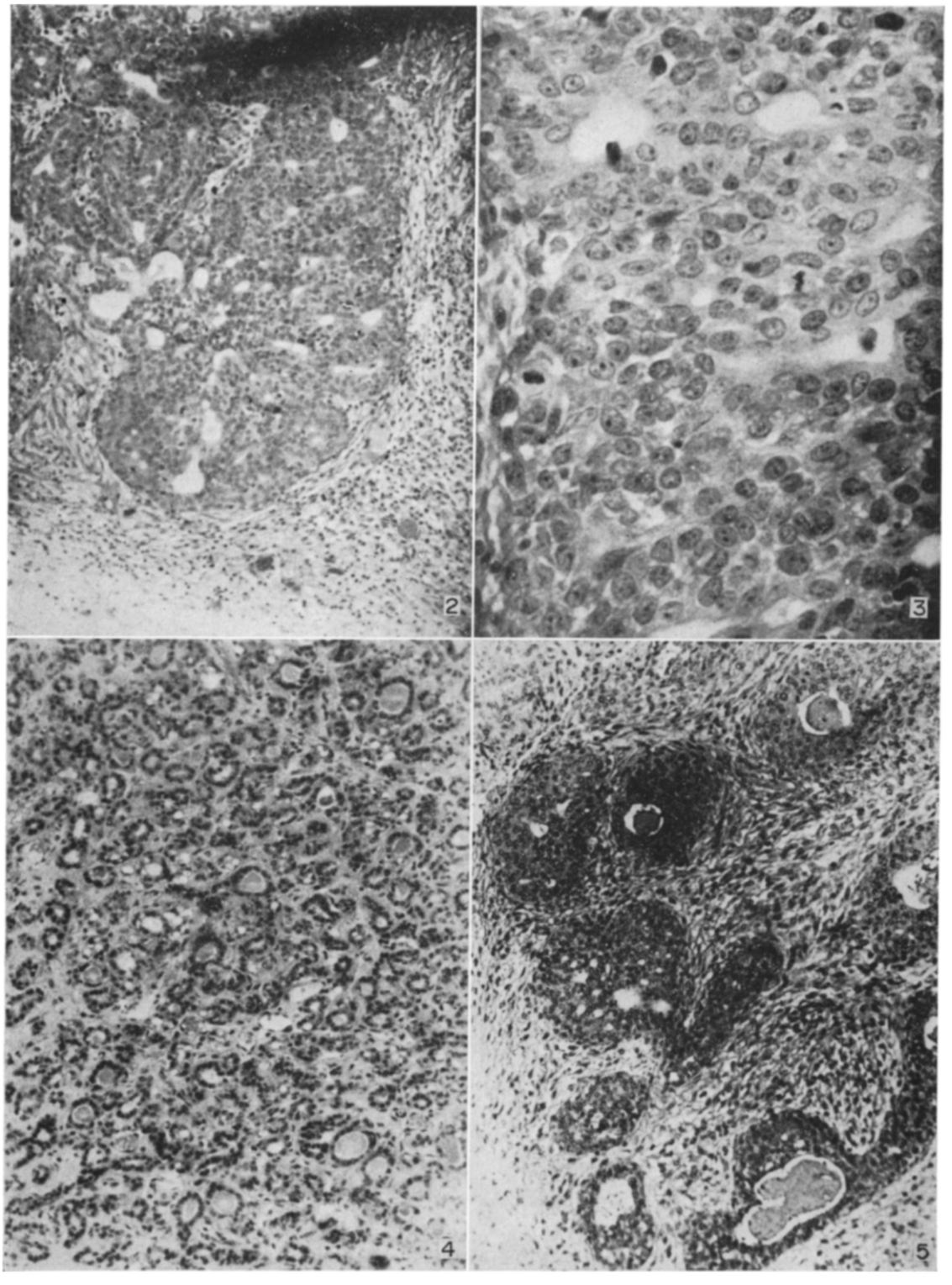

Fig. 2. *Rat* 33 × 125 (690201) *Typical papillary adeno-carcinoma.*

Fig. 3. *Rat* 33 × 400 (690201) *The same adeno-carcinoma.*

Fig. 4. *Rat* 8 × 125 (60.04.02) *Fibro-adenoma.*

Fig. 5. *Rat* 2 *Ambiguous tumour with appearance of comedo-carcinoma.*

(to face p. 606)

Table 3. Pathological aspects of mammary tumours

Histology		Group I	Group II	Group III	Group IV	Group V	Group VI
Maligrant tumours	Adeno-carcinomas	7	23	4	29	1	0
	Undif.carcinomas	0	10	0	6	0	0
Histologically ambiguous tumours		2	19	8	7	0	0
Benign tumours	Adenomas	19	14	16	16	0	2
	Fibro-aderomas	8	16	27	34	1	1
	Fibromas	3	5	8	6	0	0
Total		39	87	63	98	2	3

Table 4. Average number of histologically controlled tumours per rat in each group ($\pm S/\sqrt{n}$)

Groups	Benign tumours	Malignant tumours	Histologically ambiguous tumours	Total
Group I	2·0 ±0·2	0·46±0·1	0·13±0·1	2·59
Group II	1·75±0·3	1·65±0·2	1·0 ±0·2	4·40
Group III	2·5 ±0·5	0·20±0·1	0·4 ±0·1	3·10
Group IV	2·8 ±0·3	1·75±0·3	0·35±0·1	4·90

Table 5. Number of animals bearing malignant or benign tumour, or both

	Group I	Group II	Group III	Group IV
Malignant tumours only	0	0	0	4
Benign tumours only	6	2	17	2
Both malignant and benign tumours	9	18	3	14
Number of lung metastases	0	4	2	3

in group III bore only benign tumours. Group IV differs from the others as it was the only group in which the animals bore exclusively malignant tumours.

The same table shows the metastases observed in this experiment. No lung metastases were observed in group I. They were observed in 4 of the 20 animals in group II (20%), in 2 animals in group III (10%) and in 3 animals in group IV (15%). If the percentages are recalculated referring only to the animals presenting malignant tumours in each group, we find similar incidence of metastases in groups II and IV (4/18 and 3/18 respectively) whereas group III showed 2 metastases from animals with malignant tumours.

Others tumours (Table 6). There was an increase in the number of differentiated and keratinizing carcinomas of the Zymball gland, behind the ears, of rats which received azathioprine. All these rats developed a significantly increased number of such tumours as compared with those in group II which received only DMBA.

Table 6. Incidence of keratinizing malpighian carcinomas from the Zymball gland

	No. of carcinomas	% of animals
Group I	11	55
Group II	2	10
Group III	8	40
Group IV	9	45

One rat of group I developed three tumours very soon after the commencement of the experiment. It died on the 178th day with fibrosarcomas and has been excluded from the group.

DISCUSSION

There was no significant difference in the date of appearance of the mammary tumours or in the total number and the histological features of those tumours in groups I and III which received azathioprine before or at the same time as the DMBA. The influence of

azathioprine may thus be summarized as follows:

If azathioprine was given before or at the same time as the DMBA and the treatment continued twice weekly, the tumours appear later than in DMBA-treated controls; they were less numerous per animal, more often benign, and had a higher incidence in the Zymball gland.

However, *if azathioprine was given from the time of appearance of the first tumour onwards*, the spread of tumours was very rapid after the appearance of the fourth tumour, the number of tumours per animal was slightly higher, and the proportion of MT was closer to that seen in the DMBA-treated control group (36% compared to 38%); the tumours again often appeared in the Zymball gland (45% of animals as compared to 10% in the DMBA-treated controls). But, for each group, there was a similar incidence of HAT which was much lower than in the DMBA-treated animals.

None of the rats in group I had lung metastases, but in group II, the MT seemed to be more often metastatic (66% with lung metastases as compared to 22% in the DMBA-treated controls); in group IV, the ratio of metastases to the lung was about the same as in the DMBA-treated controls. One point seems to be of interest: the mean number of BT per rat is about the same for all groups: azathioprine thus seems to have an effect only on the ratio of MT and HAT or on the diffusion metastatic process.

The effect of azathioprine on the growth of tumours induced in the mouse was studied by Nemoto *et al.* [2]. Treatment of animals bearing either skin tumours induced by methylcholanthrene, or solid Erlich tumours, with azathioprine, did not modify the tumour growth if the treatment was started at first appearance of tumours. However, the animals were only treated and observed for 7 to 15 days. If the azathioprine was given before the carcinogen, some effect was noted. In this case, a higher percentage of induced tumours were malignant in the azathioprine-treated animals than the controls for the MCA-induced tumours (39% as compared to 18%). Similarly, the solid Erlich tumours grew faster, the mean tumour weight in the treated animals at 14 days being 1·5 time greater than that in the untreated controls. This suggests that depression of the immune response of the host potentiates the activity of a carcinogen.

The modifying action of azathioprine might be due to: (1) an effect upon the hormonal status of the host. The hormonal dependence of the mammary tumours induced by DMBA is well known [3–5]. We have found no significant modifications in the oestrus cycle of rats continuously treated with azathioprine and such treated rats were able to produce as many offspring per litter as untreated rats; (2) an effect on the immunological status of the host. A study by Wylie [6] led him to the conclusion that "rats normally do not react immunologically against their own tumours in a grossly measurable way". Similarly, Wagner and Haughton [7], studying MCA-induced tumours, concluded that "prolonged immuno-suppression by ALS has little or no effect on the incidence or latency of tumours induced in mice by a standard dose of MCA". It seems that a most important point indicated by these experiments is that the action of the immuno-suppressive agent on the tumour inducing process depends on the moment at which it is given in relation to the carcinogenic stimulus.

It is possible that the effects seen will be quite different if the immuno-suppressive agent is given during the pre-induction, induction or dissemination phases. The nature of the immuno-suppressive agent used is probably also of considerable importance.

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The Macromolecular Metabolism of Ribosomal Precursor RNA as a Common Target of Different Antitumor Drugs

UMBERTO TORELLI, GIUSEPPE TORELLI and CARLO MAURI

Institute of Medical Pathology, University of Modena, Italy

Abstract—*The ability of mechloretamine, vincristine and daunomycin to interfere with methylation and cleavage of ribosomal precursor RNA has been tested* in vitro *in PHA-stimulated human lymphocytes. The cells were incubated with uridine-^3H and ^{14}C-methyl-methionine for short periods, then the drugs were added to the medium and incubation continued for several hours. Lymphocyte RNA was extracted by a hot phenol method and analyzed on sucrose gradients. All the drugs studied caused a marked decrease in the number of radioactive methyl groups incorporated in ribosomal and transfer RNA. Accumulation of ^3H-uridine radioactivity was observed in 32S–45S RNA. It is concluded that macromolecular metabolism of ribosomal precursor RNA is a common target of different antitumor drugs.*

THE LAST few years have seen a marked progress in the knowledge of the macromolecular metabolism of RNA in mammalian cells, both normal and malignant. The macromolecular events which take place from the synthesis of the 45S ribosomal precursor RNA to the formation of the two ribosomal RNA subunits have been elucidated to a considerable extent [1]. Two points have a particular relevance to our present study: the first one is the demonstration that inhibition of protein synthesis, induced by drugs or by aminoacid deprivation, rapidly and markedly interferes with these events [2–4]. The second one is the observation that in acute leukemia blast cells synthesis and processing of 45S RNA seem to be impaired: the majority of these cells are unable properly to methylate and cleave the 45S molecule [5,6]. This observation, coupled with the demonstration that in asparagine-dependent cells one of the earliest metabolic effects of a well known antileukemic agent, l-asparaginase, is the reduction in the rate of synthesis and processing of ribosomal precursor RNA [7], suggests that the macromolecular metabolism of ribosomal precursor RNA may be a common target of several antileukemic drugs.

A group of experiments has therefore been performed in order to study the effect of different antitumor drugs such as mechloretamine, vincristine and daunomycin on the macromolecular metabolism of ribosomal precursor RNA.

Accepted 3 May 1972.

MATERIAL AND METHODS

All our experiments were performed on isolated, phytohemagglutinin (PHA)-stimulated human peripheral lymphocytes. To separate the lymphocytes from granulocytes, heparinized blood from normal donors was sedimented by gravity and the supernatant was filtered through nylon filters. Ten milliliters of lymphocyte cultures were set up in Minimal Essential Medium, with 20% of autologous plasma. The details of our methods have been already reported [8]. After 60 hr of exposure to PHA (Wellcome, Reagent Grade), uridine-5-^{3}H and ^{14}C-methyl-methionine were added at a final concentration respectively of 10 μ and 2 μCi/ml. After time intervals varying from 30 min to 3 hr, the various drugs were added and the incubation continued for 3-hr periods. The cells were then harvested by chilling on frozen saline and centrifugation at 1200 rev/min in a refrigerated centrifuge. Because of the low number of lymphocytes available in each experiment, unlabelled RNA from KB cells was used as carrier. Five to 10×10^7 lymphocytes were extracted together with 1 to 2×10^8 KB cells in each experiment.

RNA was extracted by the hot phenol-metaeresol method already described [8]. Centrifugation was carried out for 15 to 18 hr on 5 to 20% sucrose gradients in 0·01 M Tris buffer pH 7·4 with 0·1 M NaCl at 15,000 to 18,000 rev/min in the SW 25·1 rotor of a Beckman L50 ultracentrifuge. Forty-five fractions were obtained from each gradient with an ISCO gradient analyser. Radioactivity was determined on the fractions with a Packard Tricarb liquid scintillation spectrometer.

RESULTS

In all our experiments, the rate of RNA synthesis in growing lymphocytes was markedly reduced by the exposure of the cells to mechloretamine, vincristine and daunomycin. This effect is known for all these drugs and does not require further illustration. The sedimentation analysis of labelled RNA showed important changes in the distribution of the radioactive precursor after the action of the drugs. Figure 1 shows the pattern of radioactivity obtained from normal PHA-stimulated lymphocytes exposed for 3 hr to uridine-5-^{3}H and ^{14}C-methyl-methionine. The optical density and the radioactivity profiles are clearly superimposable, the peaks of both ^{3}H and ^{14}C radioactivity being coincident with the optical density peaks at 4S,18S and 28S. Figure 2 shows the distribution of radioactivity observed when cells exposed for 30 min to the two radioactive precursors were further incubated for 3 hr with mechloretamine at a concentration of 10^{-3} M. Two main events are apparent: the almost complete lack of labelled methyl groups in the two ribosomal subunits and the accumulation of

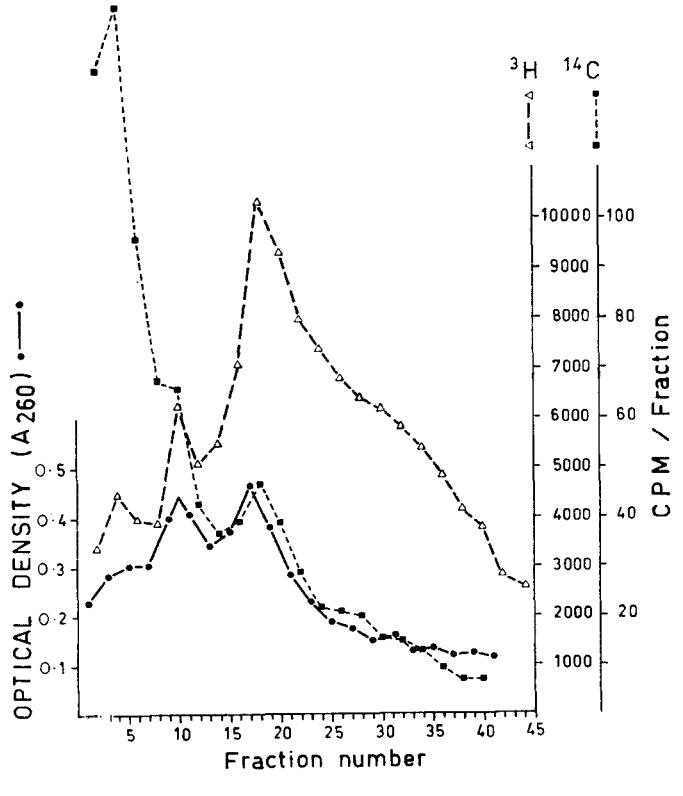

Fig. 1. Sedimentation pattern of labelled RNA from PHA-stimulated lymphocytes incubated for 3 hr with uridine-^{3}H (10 μCi/ml) and ^{14}C-methyl-methionine (2 μCi/ml). The optical density profile in this and in the following figures represents mainly KB cell RNA. Gradients were centrifuged at 15,000 rev/min for 14 hr.

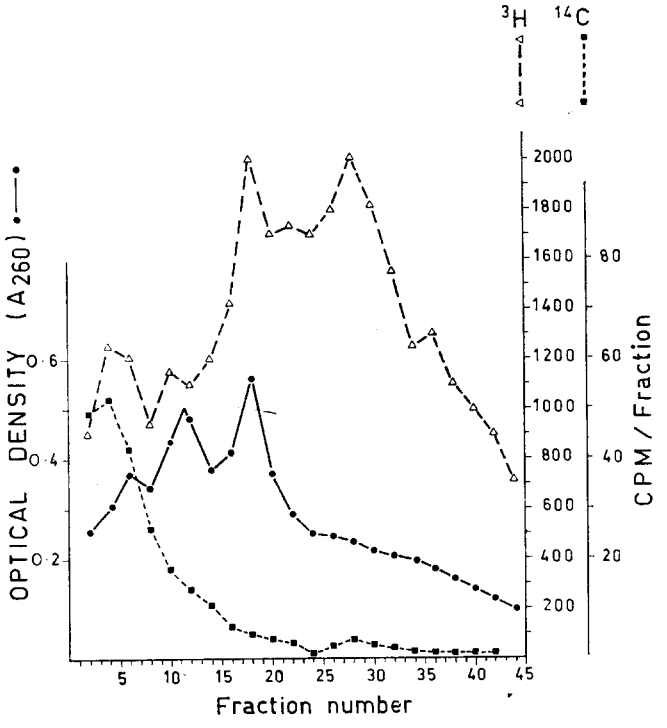

Fig. 2. Sedimentation pattern of labelled RNA from PHA stimulated lymphocytes. After 30 min of incubation with ^3H-uridine and ^{14}C-methyl-methionine, mechloretamine was added to the medium to a concentration of $10^{-3}M$, and the incubation continued for 3 hr. Centrifugation at 15,000 rev/min for 14 hr.

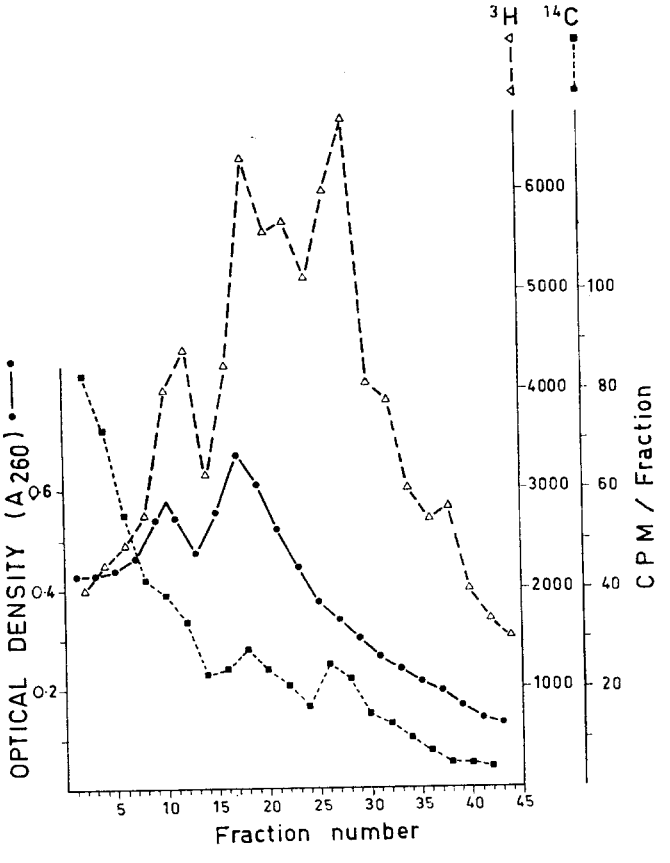

Fig. 3. Sedimentation pattern of labelled RNA from PHA-stimulated lymphocytes. After 30 min of incubation with ^3H-uridine and ^{14}C-methyl-methionine, vincristine was added ($10^{-4}M$) and incubation continued for 3 hr. Centrifugation at 15,000 rev/min for 14 hr.

^{3}H-uridine radioactivity in the 32S to 45S region of the gradient. Figure 3 shows the pattern obtained with RNA from the same number of lymphoblasts exposed for 30 min to the two radioactive precursors and then incubated for 3 hr with vincristine 10^{-4} M. Inhibition of methylation was less pronounced, but the accumulation of large amounts of radioactivity in the 45S region is quite evident. Figure 4 shows the RNA sedimentation pattern obtained under the same experimental conditions from lymphoblasts treated with daunomycin (2 μg/ml). Complete inhibition of ribosomal RNA methylation, lack of definite radioactive peaks at 18S and 28S and accumulation of the label in 32S to 45SRNA were observed.

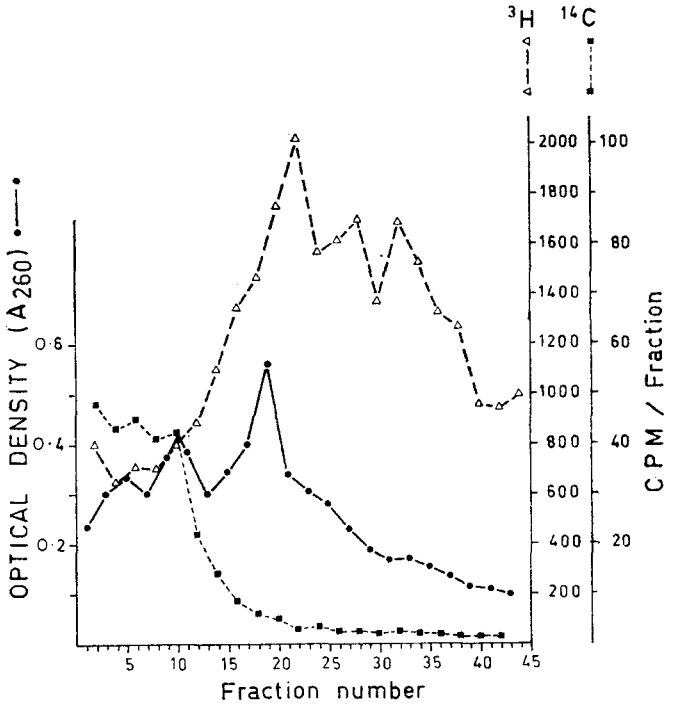

Fig. 4. Sedimentation pattern of labelled RNA from PHA-stimulated lymphocytes. After 30 min of incubation with ^{3}H- uridine and ^{14}C-methyl-methionine, daunomycin was added (2 μg/ml) and incubation continued for 3 hr. Centrifugation at 15,000 rev/min for 14 hr.

DISCUSSION

It is now generally accepted that ribosomal RNA is synthesized in the nucleolus as a 45S molecule which contains the sequence for one 28S molecule and one 18S molecule, accounting for about 50% of the molecular weight [1,9]. The 45S molecule is partially methylated and cleaved to a 32S and a 18S molecule, whereas the unmethylated portion is rapidly degraded. The 32S RNA is then converted to 28S RNA by cleavage of a small unmethylated portion. Methylation and cleavage occur at very high rate in rapidly proliferating cells, the average life of the 45S molecule being about 15 min in HeLa cells [10].

Our results indicate that the macromolecular metabolism of the ribosomal precursor RNA is affected to a considerable extent by the exposure to mechloretamine, vincristine and daunomycin. Although the patterns obtained are not identical, at least two early effects appear to be common to the drugs studied: the reduction of the rate of methylation and the reduction of the rate of cleavage of the 45S RNA.

The details of the mechanism through which these drugs act on preribosomal RNA metabolism await explanation. Nitrogen mustard causes direct inhibition of protein synthesis [11], and this action could explain the detrimental effect on synthesis and processing of ribosomal precursor RNA. In fact, maturation of 18S and 28S seems to occur for a while after addition of the drug to the culture medium, and this is in keeping with the experimental observations that any immediate requirement for ribosomal protein during the ribosomal RNA maturation may be satisfied by a pool of pre-existing ribosomal protein [12]. Vincristine too has been shown to inhibit protein synthesis [13], but its effect on maturation of ribosomal precursor RNA is presumably due to a more specific mechanism. In fact, the appearance of a definite peak of radioactivity at 18S in the presence of accumulation of radioactive 32S RNA apparently indicates that inhibition of the processing of the 45S RNA occurs after the cleavage of the 18S RNA. Our results therefore confirm the previous observations of Wagner and Roizman [14], suggesting that Vinca alkaloids affect both synthesis of 45S RNA and processing of the molecule after cleavage of 18S RNA. Finally, the extremely rapid and complete inhibition of the appearance of 18S and 28S radioactive

RNA after contact with daunomycin strongly suggest that the activity of this drug on pre-ribosomal RNA processing is not mediated through inhibition of protein synthesis but is due to a direct action on the ribosomal precursor molecule.

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Inflammation and Tumor Promotion: Selective Protein Induction in Mouse Skin by Tumor Promoters

JOHN D. SCRIBNER* and R. K. BOUTWELL

McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wisconsin 53706

Abstract—*Low doses of the tumor promoter* 12-*tetradecanoylphorbol*-13-*acetate evoke a selective increase of certain protein fractions in mouse skin cytosol. Phorbol*-12,13-*dibutyrate and phorbol*-12,13-*dimyristate have comparable promoting activities and produce a similar protein enhancement at comparable doses, although the dibutyrate is* 200 *times more inflammatory than the dimyristate. This event is not produced by inflammatory doses of acetic acid, a poor promoter, nor by the tumor initiators* 7,12-*dimethylbenz* [*a*] *anthracene or N-acetoxy*-2-*acetamidofluorene.*

INTRODUCTION

MUCH research in hepatocarcinogenesis by azo dyes and skin carcinogenesis by polycyclic aromatic hydrocarbons has been concerned with covalent binding of the carcinogen to protein in the target tissue [1, 2]. In both systems, impressive correlations have been reported between carcinogenesis and binding of compound to a specific soluble protein fraction in the tissue. With the coming of the Central Dogma of Molecular Biology there has been an increasing tendency to dismiss protein binding as an important factor in chemical carcinogenesis. The initiation–promotion system in mouse skin reminds us, however, that although attack on DNA may indeed be the initial critical event in tumorigenesis, there remain further steps before the initiated normal cell is eventually manifested as a colony of tumor cells[3]. These further steps may well consist of or be initiated by reversible alterations in the protein content and synthetic pattern of the cell. For this reason it was of interest to electrophoretically characterize the soluble proteins of mouse skin treated with a tumor promoter and seek a change uniquely associated with the promotion process. The availability of different natural and synthetic esters of phorbol with widely varying promoting and inflammatory potencies makes possible such a search[4].

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MATERIAL AND METHODS

Female CD–1 mice (Charles River Mouse Farms) 8 weeks of age were used in all experiments. Groups of 4 to 6 mice were shaved on each flank, with a narrow strip of hair left down the middle of the back. Each mouse then received both test compound and control vehicle. In this way, essentially identical gel electrophoresis patterns were easily obtained when the two flanks received identical treatment. Such was only occasionally the case when skins from separate groups of mice were compared. Repeated experience indicated that the two sides of the back responded as if totally isolated from each other.

After the mice were killed by cervical dislocation, the skins were removed and frozen in liquid nitrogen. The hypodermis was then scraped off, the skin minced and suspended in ice-cold 0·9% saline (1 ml/sample), pooled and homogenized for 30 sec with a Polytron homogenizer (model PT 10 OD, setting 6). The homogenate was centrifuged at 16,000 × **g** for 30 min, and the supernate mixed with 0·1 volume of 0·01% bromphenol blue solution and 0·9 volume of sucrose solution (40 g/100 ml solution). 0·2 ml of this mixture was then placed on a polyacrylamide gel. Seven per cent of acrylamide separation gels were prepared by the method used by Tasseron *et al* [2]. Five per cent of gels were prepared simply by altering the amounts of water and acrylamide stock solution appropriately. After the separation gels had polymerized, stacking gels were added (0·2 ml of the following mixture: 1:1:2:4 ratios of 4 mg riboflavin/100 ml water; 48 ml 1N HCl-5·98 g Tris and 0·46 ml tetramethylethylene, diamine/100 ml solution; 10·0 g acrylamide and 2·5 g methylene(bis)acrylamide/100 ml solution; 40 g sucrose/100 ml solution) and polymerized by ultraviolet light. The tray buffer was that used by Tasseron *et al* [2]. Gels were stained for 10 min in 0·1% amido black in 7% of acetic acid, destained by washing in 7% of acetic acid, and scanned in a Canalco microdensitometer connected to a Sargent SRL recorder.

RESULTS

When 2 μg of 12–0–tetradecanoylphorbol–13–acetate (TPA) [4] was applied to the backs of mice, all of the protein fractions found on gel electrophoresis were greatly increased, but near the top of the gel were found two strong bands only faintly present (sometimes undetectable) among the soluble proteins obtained from untreated skin. This dose of TPA given twice weekly induced papillomas in 50% of mice treated for 8 weeks after being given a single topical dose of 51 μg of 7,12-dimethylbenz[a]-anthracene (DMBA) (Fig. 1). Similarly, one such dose of TPA induces massive edema within 3 hr, followed at later times by greatly increased incorporation of tritiated thymidine, cytidine and leucine [5, 6]. Within a few days there is marked hyperplasia [6]. Further investigation showed that the dose of TPA could be lowered sufficiently to eliminate the edema and the general increase in protein without eliminating the induction of these two sharp bands. Increases remained in certain other specific fractions, but the response of these two bands was unfailingly reproducible, whereas the remainder of the protein spectrum varied between groups of mice. These bands will be called 'enhanced proteins' in the remainder of the text.

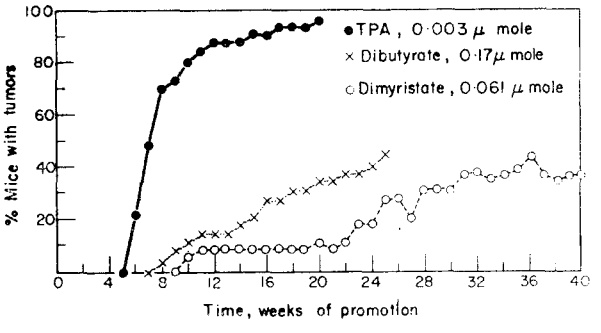

Fig. 1. Tumor promotion by esters of phorbol. All mice received a single dose of 51 μg *of DMBA. Promoters were given twice weekly in* 0·2 *ml acetone. TPA* = 12-*O-tetradecanoyl-phorbol-*13*-acetate; dibutyrate = phorbol-*12,13*-dibutyrate; dimyristate = phorbol-*12,13*-dimyristate.*

Figure 2 shows a representative electrophoretogram from a 7% gel obtained after treating mice with 0·12 μg of TPA in 50 μl of acetone on one flank. The enhanced proteins are clearly visible 2 mm from the origin, with relatively little variation from control elsewhere on the gel. On a 5% gel, the enhanced proteins are clearly separated from the origin (Fig. 3), and their uniqueness more readily demonstrated. The samples shown in this figure are from a different experiment from that shown in Fig. 2. We have found that the maximum level of enhanced proteins is attained within 3 hr of treatment, with a large decrease by 6 hr (Fig. 4).

Experiments with cycloheximide indicate that production of the enhanced proteins consists of new synthesis, rather than modification of pre-existing protein. Mice were given 0·5 μg TPA on one flank, acetone only on the other flank, and were injected i.p. with 5 mg cycloheximide,

which has been found to effectively suppress protein synthesis in mouse skin [7]. The mice were killed either 1 hr after treatment, or were given a second injection of cycloheximide 1½ hr after TPA treatment and killed after a total elapsed time of 3 hr. In either case, induction of the enhanced proteins was suppressed completely. In a 3-hr experiment the electrophoretograms from the treated and control sides of mice which had received cycloheximide were identical, whereas for animals given saline injections the enhanced protein fractions from the TPA-treated skins contained 2·9 times as much protein as the corresponding, featureless areas in the electrophoretograms from acetone-treated skin. However, when mice were treated with

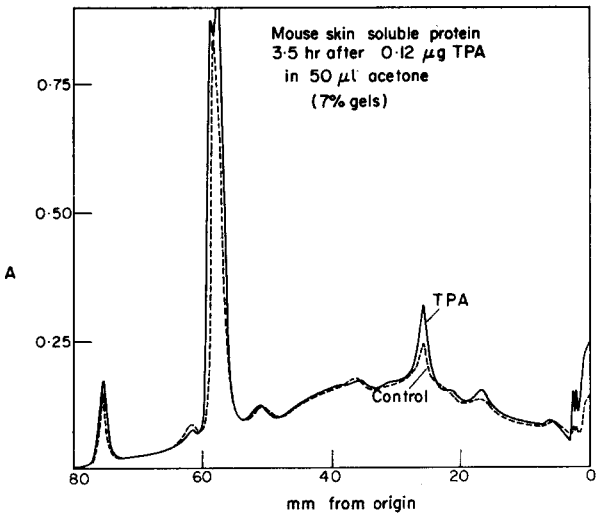

Fig. 2. Gel electrophoresis of mouse skin soluble protein. The control sample was obtained from acetone-treated skin. The 'enhanced proteins' are approximately 2 mm from the origin.

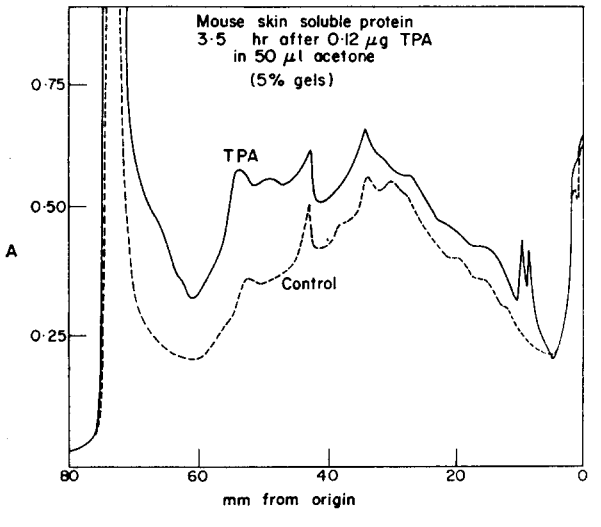

Fig. 3. Gel electrophoresis of mouse skin soluble protein. Similar to Fig. 2, but a different group of mice, and lower gel concentration. The 'enhanced proteins' are approximately 10 mm from the origin.

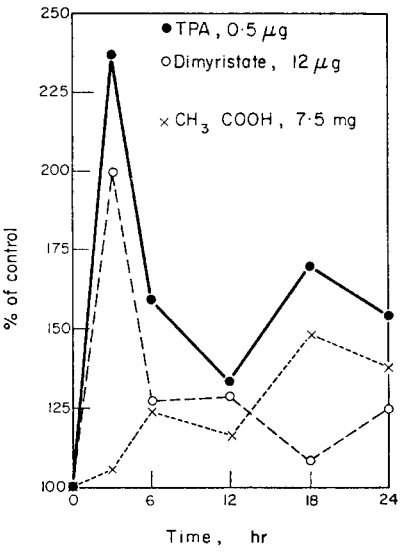

Fig. 4. Kinetics of enhanced protein induction. Each compound was applied to the right flanks of mice in 50 μl acetone. The ratio given is for peak heights (compare with Fig. 2).

0·12 μg TPA on one flank, given 100 μCi of ^{3}H-leucine 3 hr later, and killed after 30 min of incorporation, no clear peaks of activity could be found corresponding to the enhanced proteins observed by staining, even though extensive incorporation was evident in other portions of the gel. Thus it appears that the enhanced proteins are produced by new synthesis, which begins soon after treatment and ceases within 3 hr.

This protein enhancement is not unique to TPA. Other esters of phorbol also have inflammatory and promoting activity [4]. Because these activities are not correlated, it was of interest to find out what relationship exists between promoting activity and the ability to induce protein enhancement. Table 1 compares the dosage required to induce synthesis of enhanced protein with the tumor-promoting abilities of these esters (TD_{50} = total dose required to produce papillomas in 50% of survivors) and with published data on their inflammatory potencies (ID_{50} = dose required to evoke observable ear redness in 50% of the mice treated, observed 24 hr after treatment) [4, 8]. The doses required to promote tumorigenesis with these compounds appear much more closely related to the doses required to induce protein enhancement than they do to inflammatory potency. Thus we might infer that promotion is not critically dependent upon inflammation, although inflammatory potency may contribute [9].

Acetic acid is an inflammatory agent [10] with little or no promoting ability. We compared its promoting activity and effect upon the

Table 1. Promoting, inflammatory and protein-enhancing properties of phorbol esters

Compound	ID_{50} (mμmole/ear[4])	Enhanced protein dose mμmoles/5 cm^2)	TD_{50} (μmole)
TPA	0·016	0·2	0·05*
Phorbol-12,13-dibutyrate	0·067	4	9·7*
Phorbol-12,13-dimyristate	14·0	15	ca. 6*

Abbreviations are defined in the text.

*See Fig. 1 and also ref. [4].

enhanced proteins with those of the phorbol derivatives. After the usual initiation with DMBA, several experiments with varying dose schedules failed to produce tumors. When 2·5 mg of acetic acid in 50 μl of acetone was applied to the flanks of mice, no change in the pattern or amount of protein after electrophoresis was noted (mice killed 6 hr after treatment). When 7·5 mg was applied, the results shown in Fig. 4 were obtained. DMBA also fails to induce a response in this system (12 μg applied to one flank, killed after 4 hr). N-Acetoxy-2-acetamidofluorene, a potent local carcinogen in rats [11] and a tumor initiator in mouse skin at a dose of 50 μg [12], was also tested and found to be inactive (50 μg on one flank, killed after 4 hr).

DISCUSSION

A long-sought goal in the study of chemical carcinogenesis has been the identification of some early event which is unique to carcinogens among a larger class of compounds with similar structures or acting on the same tissue. The discovery of such an event would offer both a method of rapid assay for tumorigenic activity and enlightenment upon the nature of tumorigenesis. Use of the two-stage system of tumor induction in mouse skin permits one to break down the tumorigenic process into steps of initiation and promotion, and to search for events unique to agents effective in only one of these steps. The effect reported here has characteristics which suggest that it may be useful in the study of tumor promotion. It is particularly important to note that this response is not the response of tumor cells nor even of initiated cells, but rather of normal mouse skin. Thus, it cannot be viewed as a change occurring in potential tumor cells, but as a change in normal cells which, if occurring in cells carrying a permanent genotypic error due to a carcinogen, might permit potential tumor cells to express their new identity.

We have sought to indicate the possible uniqueness of this event by comparing the influence of promoting and non-promoting agents, and by comparing its occurrence with overall protein synthesis, whether determined by electrophoresis or ^{3}H-leucine incorporation. The fact that, with low doses of TPA, this effect can be produced separately from a general increase in protein synthesis, indicates that it is separate from such a general increase. Also, the early peak in protein enhancement precedes the peak of ^{3}H-leucine incorporation following administration of TPA or croton oil by at least 15 hr [5, 13]. This difference is reflected in Fig. 4, in which phorbol dimyristate (non-inflammatory at the dose indicated) and acetic acid can be compared. The response to the phorbol ester is qualitatively like that to TPA, but the only comparable response after acetic acid treatment did not appear until 18 hr, coincident with a general increase in protein. Hence, the production of enhanced proteins not only is different from the general protein synthesis detected by ^{3}H-leucine incorporation, it is also separate from the inflammatory process. Consideration of the data presented here and elsewhere even raises the possibility that inflammation is counter-productive to tumor promotion. Previous work argues both for and against this proposition. Cantharidin had an anticarcinogenic effect when given repeatedly in alternation with hydrocarbon carcinogens [14, 15], but acted as a promoter when given repeatedly after a single dose of DMBA [13]. Experiments performed in Troll's laboratory showed that direct attack on proteolytic enzymes associated with inflammation reduced the promoting effect of TPA [16]. Attempts to correlate the anti-inflammatory activities of a series of steroid hormones with inhibition of promotion have had doubtful success [17].

The role of tumor promotion remains unclear,

although Hennings and Boutwell have already pointed out that the ability to effect hyperplasia is not sufficient to produce promotion [13]. A model of the initiation process suggests a role, however: let us assume that initiation consists of introducing a mutation into an area of the genetic material which is normally masked during the functional life of the cell. Only an occasion of demasking this area during the interval between cell divisions will enable the initiated cell to become a latent tumor cell. The enhancement of specific protein fractions by tumor promoters may be explained by a selective demasking of certain areas of the genome. If a potentially tumorigenic alteration lies within one of these regions, repeated demasking may produce enough alteration in the cellular *milieu intérieur* to make this a permanent change, i.e. a tumor cell. If the repeated demasking is accompanied by the additional stress of hyperplasia, breakdown of tissue homeostasis is even more likely, favoring conversion of the initiated cells into latent, then growing tumor cells.

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Influence of Hormones, Poly I:C, Polyvinylpyrrolidon and β-Stimulators on *in vitro* Infection with Murine Leukemia Virus*

P. EBBESEN and J. HESSE

Laboratory of Tumor Virus Research, Institute of Medical Microbiology, University of Copenhagen, 22 Juliane Maries vej, DK-2100 Copenhagen, Denmark

Abstract—*The influence of various compounds on* in vitro *infection with murine leukemia virus was investigated using plaque development in the* XC-*test as indicator of infection.*

Insulin was a strong inhibitor, while cyclic AMP, glucagon and T liothyronine natrium NFN were without influence. AMP, glucagon, isoprenalin and T liothyronine natrium NFN, however, partly abrogated the insulin inhibition when administered together with that hormone.

The interferon-inducer poly I:C was a strong inhibitor. Cyclic AMP, glucagon and isoprenalin counteracted the effect of poly I:C. Polyvinylpyrrolidon inhibited at a high concentration and enhanced at lower concentrations.

Isoprenalin (β-stimulator) increased the number of foci slightly, while propanolol (β-inhibitor) significantly depressed the focus count.

The results are interpreted in terms of a possible role for cyclic AMP and a possible importance of the β-receptor sites for compounds influencing the process of infection.

INTRODUCTION

TREATMENT with estrogen cortisone and the interferon-inducer poly I:C has profound effects on the outcome of malignant processes, including those following *in vivo* infection with leukemia virus [1–8]. *In vivo* experiments do not distinguish between those effects of hormones and poly I:C on oncogenesis that are secondary to altered immune reactivity [9, 10] and those which derive from interaction with the transforming cells. To study the influence of these compounds on infection with murine leukemia virus in an environment free of immune competent cells we have used *in vitro* infection of murine fibroblasts as the test systems. Additional hormones and the membrane-active polymer polyvinylpyrrolidon (PVP) [11] have been included in the tests to further elucidate factors influencing the developments of infectious centers.

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MATERIAL AND METHODS

Virus

A BALB/c tropic [12] murine leukemia virus B_1 isolated from a BALB/c reticulosarcoma [13] was harvested from the fourth passage in BALB/c embryo fibroblast cultures.

Rauscher virus was also harvested from BALB/c embryo fibroblast cultures.

Minimum Essential Medium, Eagle (MEM) with Hank's balanced salt solution pH 7·2 was used as diluent for all compounds. Cells were grown in the same medium supplemented with 10% of fetal calf serum.

The compounds tested and the doses used are in Table 1.

Table 1.

Compounds	Doses used per 2 ml MEM
Insulin Leo, Neutral Nordisk Insulin Laboratorium Ved Stadion 2, DK-2820 Gentofte, Denmark	16 units
N^6—2^1O—dibutyryl—A 3·5—MP (Cyclic AMP) Cat. No. 15205 NAAT Boehringer, Mannheim, Western Germany	1 mg
Glucagon, Novo Industri A/S Fuglebakkevej 115, DK-2200 Copenhagen, Denmark	50 μg
Tertroxin (liothyronine natrium NFN) Glaxo Laboratories Ltd. Greenford, Middlesex, England	5 μg
Inj. Isoprenalini sulfatis 0·1%	50 μg
Estradiolbenzoat, research product Løvens Kemiske Fabrik Industriparken 55, DK-2750 Ballerup, Denmark	50 μg
Pulvis hydrocortisonum Ph. Nord. 63	50 μg
Poly I: poly C code 11–321 lot 8 Miles Laboratories, Research Products Division INC Kanhahee, ILL 60901	1 unit
Polyvinylpyrrolidon K15 (PVP) MW 10,000 Fluka AG, Buchs SG, Switzerland	200 mg = 10^0 20 mg = 10^{-1} 2 mg = 10^{-2}
Inderal (propanolol chlorid) Imperial Chemical Industries Limited ICI Pharmaceuticals Division, Alderley Park Macclesfield, Cheshire, England	50 μg

Testing

Testing of compounds for influence on infection was performed by the following modification of the XC-test of Rowe *et al.* [14].

Primary cultures were established from 16-day-old BALB/c embryos. After a week, secondary cultures were seeded in Falcon plastic dishes with a diameter of 60 mm and kept in a 5% CO_2 atmosphere at 37°C. The following day the subconfluent cultures were incubated with 50 μg DEAE-dextrane MW 2×10^6 in 2 ml MEM for 60 min at 37°C. After washing in phosphate buffered saline with Ca^{2+} and Mg^{2+} (PBS) incubation was continued with the compound to be tested in 2 ml MEM for 60 min at 37°C. Following washing in PBS, 50 or 100 XC-units of virus were added in 5 ml of MEM with 10% fetal calf serum. Medium was changed every 24 hr. On the 5th day after infection the cells were killed with u.v. light and overlayered with 10^6 XC-cells. On the same day control dishes were trypsinized and the cells counted in order to exclude an effect on cell growth of the different compounds used. The number of plaques in the XC-culture present 3 days later was our measure of infection. Three replicate cultures were used for each test. Each test was redone at least twice. Results of different experiments were averaged using the 50 and 100 plaque norms.

RESULTS

Insulin in the dose indicated significantly reduced the number of foci, without influencing the cell count (Table 2). The plaque count was unchanged by insulin solvens. Increase of insulin concentration to 40 units/2 ml MEM resulted in a slower multiplication of the infected cells. Cyclic AMP, glucagon and Tertroxin had no certain effect when used

alone. The same compounds, however, were capable of partly abrogating the insulin inhibition when administered together with insulin. Isoprenalin, which had an enhancing effect when used alone, seemed completely to abrogate the inhibitory effect of insulin. Estrogen, estrogen solvens (alcohol) and hydrocortisone had no effect in the doses used.

Table 2. Hormone effects on in vitro *infection with murine leukemia virus*

Hormone	XC-plaques/dish
None	100
Insulin	5
Insulin solvens	100
Cyclic AMP	90
Glucagon	100
Tertroxin	60
Isoprenalin	170
Insulin + AMP	50
Insulin + Glucagon	15
Insulin + Tertroxin	15
Insulin + Isoprenalin	100
Estrogen	90
1% CH_3CH_2OH (Estrogen solvens)	100
Hydrocortisone	100

All hormone tests were redone, first with cyclic AMP added together with all virus samples and later with the hormones and virus added simultaneously. These modifications did not change the results significantly.

The tests were furthermore redone without DEAE-dextrane pretreatment and with Rauscher virus instead of B_1 virus. This modification also gave the same results as with our standard procedure.

Poly I:C nearly completely abrogated the infection. Cyclic AMP, Glucagon and Isoprenalin partly neutralized the effect of Poly I:C (Table 3).

Table 3. Influence of various compounds on in vitro *infection of secondary mouse embryo fibroblasts with murine leukemia virus* B_1

Compound	XC-plaques/dish
None	50
Poly I:C	5
Poly I:C + AMP	15
Poly I:C + Glucagon	40
Poly I:C + Isoprenalin	50
PVP 10^0	0
10^{-1}	100
10^{-2}	70
Isoprenalin (β-stimulator)	85
Propanolol (β-inhibitor)	25

PVP in high concentration, i.e., 10^0 (see Material and Methods), inhibited infection, whereas 10^{-1} doubled the number of foci and 10^{-2} was slightly enhancing.

Isoprenalin (β-stimulator) had an enhancing effect, whereas propanolol (β-inhibitor) was an inhibitor.

Infectious center counts of replicate cultures did not differ more than 15%.

DISCUSSION

Electron microscopic pictures have been interpreted as showing infection of cells with leukemia virus by phagocytosis [15]. Whatever the detail mechanism may be it requires metabolic energy. Infection furthermore occurs with some degree of specificity, since all leukemia viruses that have not been too long in laboratory passage show restricted host ranges both *in vivo* and *in vitro* [12, 16]. In the present experiments we have used a field isolate [17] virus B_1 [13] and cells from mice of its host strain.

Our test system differs from *in vivo* conditions in being free of immunocompetent cells. Firstly, the primary cultures were made from 16-day-old fetus that do not have immune competence [18]. Secondly, lymphocytes are floaters and hence washed away from the cultures at medium change. Thirdly, we found that fixed macrophages harvested from the peritoneal cavity of mice do not survive beyond the first tissue culture passage with the technique used here. The results, therefore, must be considered in the frame of interaction between compound tested and cell to be infected.

The leukemia-inducing effect of infected secondary BALB/c embryo fibroblast has been demonstrated by *in vivo* inoculation [13].

Since addition of the hormone together with the virus gave the same result as incubation with the hormone followed by a wash prior to the addition of the virus the hormone effect which we are observing must have a rapid initiation and a short induction time.

Antagonism between insulin and AMP is to be expected if a lowering of the AMP level is a main effect of insulin [19, 20]. Abrogation of insulin inhibition by glucagon, Tertroxin and isoprenalin might be explained in the same way [21, 22]. All this points to infection with leukemia virus as yet another membrane process [23] where AMP may be a key substance. Most likely the AMP-dependent processes are involved in the pinocytosis. A hormone-induced alteration of membrane configuration changing the accessibility of receptor/

carrier sites might, however, also be involved [24]. Local changes in membrane surface charge are most likely responsible for the enhancing respectively inhibiting effect of polycations and polyanions on *in vitro* infection with leukemia virus [25, 26]. Future work on the interaction of polycations/polyanions and hormones/AMP should reveal whether change in surface charge is important for the hormone actions and whether AMP is involved in the polycation/polyanion effect.

Protracted estrogen treatment of intact animals has a leukemogenic effect [4, 27], and the leukemia virus present [17] is considered of etiologic importance.

The failure of estrogen to influence *in vitro* infection suggests an indirect oncogenic effect in intact animals, probably via the estrogen-induced immune depression [3].

In vivo effects of hydrocortisone on viral leukemogenesis [6] may also primarily derive from effects on the immune apparatus since we found no influence on *in vitro* development of infectious centers.

The inhibitory effect of Poly I:C *in vitro* suggests that *in vivo* protection [7, 8] is largely due to a poly I:C effect on the infected cells rather than interference with the immune apparatus [10]. Uptake of Poly I:C is probably by pinocytosis [28]. Antagonism between Poly I:C and the three compounds Cyclic AMP, glucagon and isoprenalin therefore could derive from the influence of these compounds on pinocytosis. Poly I:C initiates interferon release and production [29, 30] but has additional biological effects [31, 32]. Possible interactions between poly I:C and hormone-induced intracellular processes therefore are not necessarily related to virus–interferon interaction.

PVP like dextran can be used as a "developing agent" in hemagglutination tests [11]. It has previously been demonstrated to have a virus-inhibitory activity presumably due to membrane effects, since no virus neutralization or interferon stimulation was found [33].

That we also found an inhibitory effect at high concentration but a stimulatory effect at lower concentrations might be explained by phagocytose of PVP [34]. At high PVP concentrations the sites of phagocytose may be blocked whereas a better virus/PVP ratio at lower PVP concentrations should bring along virus.

A slightly enhancing effect after treatment with the β-stimulator isoprenalin and the significant inhibition after treatment with the β-inhibitor propanolol points to a possible role of these receptors [35, 36] or of processes elicited through these receptors for the infection. Most likely the receptor-active substances influence the pinocytosis. Studies on this problem are in progress.

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Nearest Neighbor Frequencies of Mitochondrial DNA in Spontaneous Mouse Hepatomas

O. ANTONOGLOU, A. SYMEONIDIS and J. G. GEORGATSOS

(*Laboratory of Biochemistry, Theagenion Cancer Institute, Thessaloniki, Greece*)

Abstract—*The frequencies of the* 16 *possible dinucleotides were determined in mitochondrial DNA of mouse hepatoma. The results show that more than half the dinucleotides deviate by more than* 20% *from random distribution, as is also the case with mitochondrial DNA of normal liver but there is one important difference: the CpG frequency is random in the mitochondrial DNA of normal liver, as it is in bacterial chromosome, while in the mitochondrial DNA of hepatomas, the frequency of this nucleotide deviates significantly from random, as it does in the nuclear DNA of eukaryotic cells.*

ATTEMPTS to correlate malignant transformation with structural alterations in the cellular genome have thus far been unsuccessful. In the past few years, considerable work has been done on mitochondrial DNA which is actually another site of information storage within the eukaryotic cell. Mitochondrial DNA is a much smaller molecule than nuclear DNA by at least two orders of magnitude. This is perhaps the main reason why more information is available about the size, shape [1], replication [2, 3] and transcription [4] of the mitochondrial genome than about its nuclear counterpart. Recently in this laboratory we were able to determine the dinucleotide frequencies of mouse liver mitochondrial DNA and found them to be much less random than the same frequencies of the respective nuclear DNA [5]. In the present communication, the frequencies of dinucleotides in mitochondrial DNA of spontaneous mouse hepatomas are presented.

MATERIAL AND METHODS

Material

Approximately one year old male mice of the pure inbred strain C3HAvy were used throughout this work [6]. We have observed with this strain an incidence of more than 70% of spontaneous hepatomas. Livers were removed from ether anesthetized animals and further processes were carried out only when a tumor was macroscopically apparent. Histologic examination of each specimen always confirmed the gross-anatomic findings.

DNA dependent RNA polymerase was prepared by the method of Weiss [7] from dried cells of *Micrococcus lysodeicticus* purchased from Sigma U.S.A.. Carrier-free orthophosphate ^{-32}P was purchased from C.E.A. France. Alpha ^{-32}P labeled nucleoside triphosphates (approximate specific radioactivity 1mCi/μmole) were prepared according to the method of Symons [8, 9]. Mitochondrial DNA was prepared by the detergent-phenol method of Chatterjee *et al.* [10] using mitochondria isolated from hepatomas [11]. The DNA was further purified as described by Antonoglou and Georgatsos [5].

Methods

Mitochondrial DNA was transcribed on ^{32}P-labeled RNA in the assay system of Weiss [7]. The isolation of the RNA product, its hydrolysis and the isolation of the 3′-nucleoside monophosphates have been described in detail by Antonoglou and Georgatsos [5].

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RESULTS

Table 1 presents the frequencies of 16 dinucleotides of RNA synthesized on a template of hepatoma mitochondrial DNA. Table 2 shows the proportion of bases in the DNA templates as estimated from nearest neighbor sequences by applying the calculations of Josse and Swartz [12]. The basic incorporation factors were derived from the data using nearest neighbor analysis as presented in Table 3. Figure 1 shows the observed nearest neighbor frequencies of the dinucleotides versus the calculated frequencies for hepatoma mitochondrial DNA, assuming that the synthesized RNA was a true copy of the DNA template. Even though only one experiment is presented, three experiments were performed, with pooled hepatomas from 10 animals. The values of the dinucleotide frequencies in the three experiments differed by less than 10% in all cases (Table 3).

Table 1. Nearest neighbor frequencies in RNA synthesized on mouse hepatoma mitochondrial DNA template, expressed in decimal proportions of 1

	Up	Ap	Cp	Gp	Sum
pA	0·049	0·152	0·061	0·062	0·324
pU	0·129	0·063	0·056	0·044	0·292
pG	0·062	0·066	0·019	0·049	0·196
pC	0·052	0·045	0·050	0·041	0·188
Sum	0·292	0·326	0·186	0·196	1·000
Sums	0·618		0·382		
A+U/G+C	=		1·62		

Table 2. Base composition of RNA synthesized on the indicated DNA templates, estimated by the nearest neighbor technique and expressed as percent of total

Base	Mitochondrial DNA Hepatoma	Liver	Nuclear DNA
A	32·4	31·5	29·8
T(U)	29·2	32·4	27·8
G	19·6	18·1	21·0
C	18·8	18·0	21·4

Table 3. Nearest neighbor frequencies in RNAs synthesized on the indicated DNA templates and expressed in decimal proportions of 1.

Dinucleotide	Nuclear DNA	Mitochondrial DNA Liver	Hepatoma	*
ApA	0·101	0·156	0·152	5
CpA	0·075	0·052	0·061	7
GpA	0·066	0·051	0·062	7
UpA	0·055	0·056	0·049	6
ApC	0·053	0·040	0·045	10
CpC	0·054	0·052	0·050	8
GpC	0·043	0·042	0·041	9
UpC	0·060	0·047	0·052	9
ApG	0·075	0·053	0·066	8
CpG	0·011	0·028	0·019	10
GpG	0·055	0·048	0·049	6
UpG	0·073	0·052	0·061	9
ApU	0·070	0·065	0·063	5
CpU	0·070	0·050	0·056	5
GpU	0·049	0·039	0·044	7
UpU	0·090	0·169	0·129	5

*Percentage of maximal deviation from the mean of three experiments with hepatoma mitochondrial DNA.

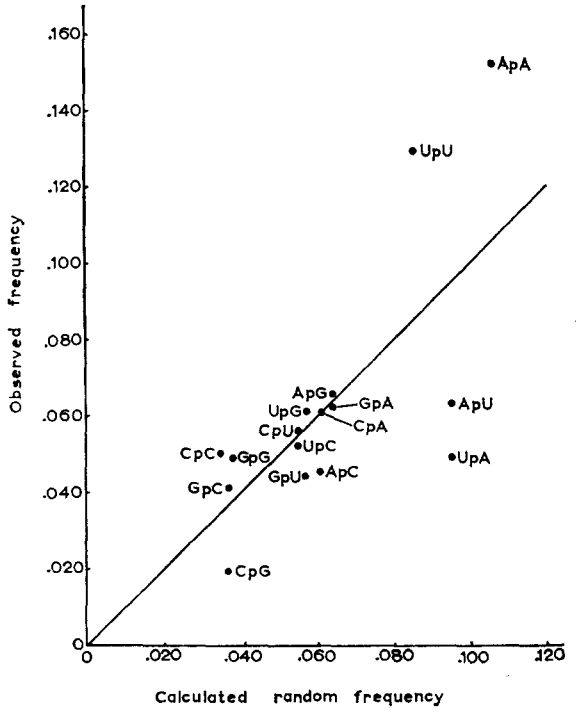

Fig. 1. The observed nearest neighbor frequencies of RNA synthesized on mouse hepatoma mitochondrial DNA template plotted against the expected random frequencies calculated from the base compositions of the samples (Table 1).

DISCUSSION

The technique of nearest neighbor analysis for the determination of base sequences in DNA was introduced by Josse *et al.* [13]. Swartz *et al.* [14] found no significant differences in the dinucleotide frequencies of nuclear DNA in four mouse tissues including a lymphoma and an ascites tumor. In this study we determined the nearest neighbor frequencies of RNA synthesized on mitochondrial DNA template of mouse hepatomas. Several investigators have already shown that when both strands of a DNA template are transcribed, the nearest neighbor frequencies of the RNA product are the same as those of the

DNA template [15]. The fact that A is apparently complementary to U and G to C, as found in our study, (Table 2) constitutes satisfactory evidence that both strands of mitochondrial DNA are indeed transcribed and consequently the dinucleotide frequencies in both, DNA strand and RNA product are the same.

As in the case with mitochondrial DNA from normal mouse liver [5], more than half of the dinucleotide sequences in mitochondrial DNA of mouse hepatomas and specifically CpC, ApA, CpG, ApC, GpU, GpG, UpA and ApU deviate by more than 20% from random as opposed to nuclear DNA where only two dinucleotides CpG and UpA fall into this category. The only significant difference between hepatoma and liver mitochondrial DNA is that in liver CpG falls within the 10% experimental error from randomness which is also the case in bacterial DNAs. In hepatoma, however, the same CpG frequency deviates by approximately 50% from random, which is very similar to the case of nuclear DNAs of eukaryotes. This, we believe, is the first significant difference found in the structure of DNA between a malignant tissue and its normal counterpart.

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Potentiation of the Antitumour Effect of Rubidomycin and Rubomycin by Non-ionic Detergents

G. C. EASTY, J. A. STOCK and ALEXANDRA UKLEJA-BORTKIEWICZ

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London SW3 6JB, England

Abstract—*The ability of rubidomycin and rubomycin to inhibit the growth of Landschütz ascites tumour in organs of the chick embryo following intravenous injection was considerably enhanced by the non-ionic detergent Triton WR* 1339 (*I*) *and related products. The greatest enhancement was observed with a dimeric analogue Gem* 30 (*III*), *while macrocyclon* (*V*), *Tween* 80, *bovine plasma albumin and bovine γ globulin gave no enhancement. None of the detergents by themselves inhibited the growth of tumours.*

Cyclophosphamide suppressed tumour growth when injected alone 48 *hr after tumour inoculation.*

THE EFFECTS of non-ionic detergents, alone or with cytotoxic drugs, upon tumour growth have been examined by a number of workers. Miller and Janicki [1] reported that Triton WR 1339 (I) displayed a cytotoxic effect *in vitro* against cells of malignant origin; cultures derived from normal cells were substantially unaffected even at a much higher (8 X) detergent concentration. The related macrocyclon (V) showed no activity against any of the cell lines. Garattini and his colleagues did not find that tumour cells were more sensitive than normal cells to Triton WR 1339 *in vitro* [2]. Their major observation, however, was that the detergent reduced the number of metastases deriving from the Lewis lung carcinoma implanted in the leg, the S180 mouse sarcoma implanted intracerebrally, and the Ehrlich ascites carcinoma, growing intracerebrally or intratibially; the growth of the primary tumour was not affected [2, 3]. On the other hand, Carter *et al.* [4] found that Triton WR 1339 and similar products, including macrocyclon, substantially enhanced the metastatic spread of a hamster transplantable lymphoma, but slightly retarded the growth of the primary tumour. Hanson and Bohley [5] noted that DL-glyceraldehyde given alone did not affect the growth of intramuscular Ehrlich carcinoma cells but partially inhibited the tumour when injected with detergents, particularly Triton X 100 (VI). No such synergism was seen when the tumour was growing intraperitoneally. Yamada and Iwanami [6] reported that pretreatment with a related non-ionic surfactant, Tween 80 (a polyoxyethylene poly-ether of sorbitan monoleate), increased the sensitivity of various strains of ascites hepatoma to nitromin *in vitro*. Similarly, Biedler and Riehm [7] found that Tween 80 potentiated the action *in vitro* of actinomycin D against an actinomycin-resistant strain of diploid hamster cells.

During the course of experiments in which the effects of macromolecular substances upon tumour growth were being explored, we observed that Triton WR 1339, and two closely related substances, enhanced the activity *in vivo* of the antitumour antibiotics rubidomycin and rubomycin. It was also noted that a well-tolerated dose of cyclophosphamide, given up to 48 hr after inoculation, strongly suppressed tumour growth. The system used was the

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Landschütz ascites tumour growing in the developing chick embryo. This is one of a number of systems in which the growth and distribution of heterologous tumour cells in the chick embryo have been studied in detail [8, 9].

MATERIAL AND METHODS

Agents

The rubomycin was kindly given (to A.U.-B.) by Professor G. F. Gause of the Scientific Research Institute for the Investigation of New Antibiotics, Moscow. "Cerubidin" brand of rubidomycin (daunomycin; daunorubicin) was purchased from May and Baker Ltd., Dagenham, and "Endoxana" brand of cyclophosphamide from Ward Blenkinsop Pharmaceuticals Ltd., London. We are indebted to the Winthrop Laboratories, Newcastle-upon-Tyne, and to the National Institute for Medical Research, London for gifts of Triton WR 1339 and macrocyclon respectively. The proteins employed were crystalline bovine plasma albumin and fraction II bovine plasma γ globulin, both from Armour Pharmaceutical Co., Ltd. Tween 80 was obtained from Koch-Light Laboratories Ltd.

Poly 30 (II), Gem 30 (III), Gem 15 (IV) and Mono 30 (VII) were prepared by the method of Cornforth *et al.* [10], except that sealed borosilicate glass combustion tubes were used in place of the steel autoclave.

Nomenclature

Mono, Gem (= gemini), and Poly refer to monocyclic, dicyclic, and mixed polycyclic structures respectively; the associated numeral indicates the average number of oxyethylene units per nucleus. Triton WR 1339 and Triton X 100 belong to the Poly and Mono series respectively.

Chromatographic behaviour of antibiotics

Thin layer chromatography of rubomycin and rubidomycin was carried out on silicic acid (Merck Kieselgel G) or cellulose (Schleicher and Schull Avicel 144). Elution with ethanol, ethanol containing 2% HCl, or ethanol containing 2% Et_3N showed that the antibiotic samples were not identical; each contained several components.

Cell suspension

Suspensions of Landschütz tumour cells, maintained in the ascitic form by passage in BALB/C mice, were prepared immediately before use as described earlier [8]. The concentration was adjusted in most experiments to 8×10^6 cells per 0·1 ml, and the suspension kept cold (ice-water).

Inoculation, treatment and post-mortem examination

White Leghorn fertilised eggs were incubated in a "Westernette" humidified incubator (Western Incubators Ltd.). The procedure followed that used in the earlier work [8], but as the relevant publication of Beveridge and Burnett [11] is not now readily available, a brief description follows. The eggs were candled on the 10th, sometimes the 11th, day of incubation, the position and direction of blood flow of a suitable vein marked on each egg, and the overlying rectangle of shell carefully cut with a carborundum wheel and removed. The exposed shell membrane and the attached chorioallantoic membrane were then made transparent by brushing on a little sterile liquid paraffin. A 0·1 ml aliquot of the tumour cell suspension was injected through a 30-gauge needle into the vein in the direction of the blood flow. The vein was revealed by illumination of the broad end of the egg with a collimated light source. The exposed membrane was covered with Sellotape and the egg returned to the incubator; eggs were not left at room temperature for more than 15 min. After an interval (usually 40–60 min) freshly prepared saline solutions of the agent or agents were injected in a similar way (0·1 ml aliquots) into a different vein or different section of the same vein. Where two agents were given, they were mixed before injection. Toxicities were assessed in non-tumour-bearing eggs. About 6 eggs were normally used in each test group. On the 5th day after inoculation, surviving embryos were dissected and examined. The body and liver weights were recorded. In a proportion of experiments, slices of liver (several from each embryo and from different lobes) were fixed in Bouin's fixative for subsequent sectioning and staining.

RESULTS

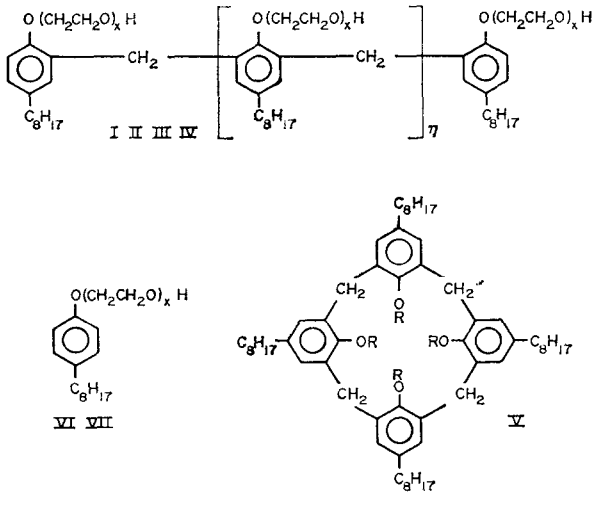
In most of the chemotherapeutic experiments, the embryos were inoculated with tumour and with the agents on the 10th day of incubation. A proportion of the embryos died during the first day from haemorrhage, more marked when a double injection was given. The approximate maximum tolerated dose of agents per 10-day embryo is given in Table 1. The corresponding values for the 11-day embryo were determined in some cases. The carcass and liver weights of normal embryos

Table 1. *Toxicity of agents administered intravenously to the chick embryo on the* 10*th day of incubation*

Agent	MTD*†	Agent‡	MTD
Rubidomycin	25 μg (45 μg)	Gem 30	3 mg
Rubomycin	50 μg (100 μg)	Gem 15	1 mg
Cyclophosphamide	0·3 mg (0·5 mg)	Mono 30	0·2 mg
Triton WR 1339	> 10 mg	BPA	> 10 mg
Poly 30	> 20 mg	BGG	> 10 mg
Macrocyclon	> 10 mg	Rubidomycin + Gem 30	20 μg + 2 mg

*Approx. maximum tolerated dose/embryo.
†Values for 11-day embryos in parentheses.
‡BPA, bovine plasma albumin; BGG, bovine γ globulin.

injected on day 10 or 11 with non-lethal doses of agents and examined on the 15th day of incubation were within the normal range, and histological examination of sections of the livers revealed no obvious abnormalities apart from small areas of necrosis and regeneration at the tips of some of the lobes receiving the highest doses of the agents. The toxicity of rubidomycin was only marginally increased by combination with Gem 30.



I; n = 0, 1, 2, 3.....(mean ~ 3); x ~ 20 (mean)
II; n = 0, 1, 2, 3.....(mean ~ 3); x = 30 (mean)
III; n = 0; x = 30 (mean)
IV; n = 0; x = 15 (mean)
V; R = $(CH_2CH_2O)_{12\cdot5\ (mean)}H$
VI; x ~ 10 (mean)
VII; x = 30 (mean)

The Landschütz tumour grows in many organs of the chick embryo following intravenous injection but most extensively in the liver. With a suitable inoculum ($5–10 \times 10^6$ cells) the liver becomes grossly enlarged after 4 to 5 days with large areas of tumour growth visible on the surface. The weight of these livers is frequently 2 to 3 times that of the livers of normal embryos of the same age (Tables 2 to 5). Examination of sections of these tumour-bearing livers revealed that half to three-quarters of the tissue consisted of tumour cells. The body weight range of the normal embryos was similar to that of the tumour-bearing embryos, treated or untreated (Tables 2 and 3). The weight of the liver therefore could be taken as an index of tumour growth, or the ratio body weight/liver weight to allow for variations in the size of the embryos. In the absence of tumour growth the ratio was between 40 and 50+, while untreated tumour-bearing animals gave a value between 10 and 20 (Tables 2–5). Intermediate ratio values corresponded well with intermediate amounts of tumour growth as assessed by histological examination. It should be emphasised that the weight of the liver alone or relative to the body weight was no more than a gross index of the amount of tumour present. Thus, tumour-inoculated embryos treated with agents could have a liver weight or ratio comparable with a normal embryo but still contain 10% or more tumour cells, as judged by histological examination.

In the early experiments rubomycin was employed, and it became clear from this initial work that Gem 30 potentiated the antitumour effect of the antibiotic in the chick embryo system. Most of the subsequent work was done with the more readily available rubidomycin; the latter was similar biologically to rubomycin, though more potent per given dose. Representative experiments are summarized in Table 2.

As can be seen in this Table, the greatest antitumour effect was given by combinations of rubidomycin and Gem 30. Neither compound alone had a significant effect at doses

Table 2. Effect of rubidomycin and macromolecules upon Landschütz ascites tumour growth in the chick embryo

Experiment	Group[a]	No. of embryos	Survivors at incubation day 11[b]	14	15	Mean wt (g)[c] of Embryo(E)	Liver(L)	Wt E / Wt L	Gross appearance of liver[d]	Estimated mean % tumour cells[e]
Ia	Normal embryos (untreated)	3	3	3	3	10·4	0·248	42		
Ib	Normal embryos (untreated)	6	6	6	6	12·1	0·256	47		
Ic	Normal embryos (untreated)	4	4	4	4	10·0	0·188	53	Normal	0
Id	Normal embryos (untreated)	5	5	5	5	9·1	0·192	47		
Ie	Normal embryos (untreated)	6	6	6	6	10·0	0·230	43		
IIa	Tumour-bearing embryos (untreated)	4	4	4	4	9·5	0·705	13		
IIb	Tumour-bearing embryos (untreated)	7	4	4	4	10·2	0·682	15		
IIc	Tumour-bearing embryos (untreated)	8	8	8	7	11·4	0·662	17	HT	>50
IId	Tumour-bearing embryos (untreated)	7	7	–	4	9·0	0·661	14		
IIe	Tumour-bearing embryos (untreated)	14	8	8	8	9·2	0·637	14		
III	15 μg rub[f]	4	4	4	4	9·5	0·560	17	T	>50
IV	12 mg Poly 30[f]	5	5	5	5	9·6	0·644	15	HT	—
V	1·5 mg Gem 30[f]	6	4	4	4	9·75	0·541	18	HT	—
VI	10 μg rub + 1 mg Triton[g]	6	6	6	6	11·7	0·438	27	T	25–50
VII	10 μg rub + 7 mg Triton[h]	5	3	3	3	11·8	0·362	33	Normal	—
VIII	10 μg rub + 1 mg Poly 30[g]	6	6	6	6	11·8	0·491	24	T	25–50
IX	10 μg rub + 12 mg Poly 30[h]	7	5	5	5	11·7	0·363	32	Normal	—
X	10 μg rub + 1 mg Gem 30[g]	6	4	4	4	10·7	0·283	38	Normal	10–25
XI	15 μg rub + 1 mg Gem 30[i]	5	3	3	3	9·7	0·230	42	Normal	5–10
XII	10 μg rub + 0·2 mg Mono 30[h]	6	5	5	4	11·7	0·541	22	T in 3/4	>50
XIII	10 μg rub + 6·25 mg Macro[g]	6	5	5	5	11·4	0·839	14	HT	>50
XIV	10 μg rub + 1 mg Tween 80[g]	6	6	6	5	11·0	0·643	17	HT	—
XV	10 μg rub + 5 mg BPA[g]	6	6	6	6	10·5	0·617	17	HT	>50
XVI	10 μg rub + 5 mg BGG[i]	6	3	3	3	9·5	0·510	19	HT	—
XVII	20 μg rub + 2 mg Gem 30[jk]	10	10	7	6	8·9	0·340	26	Normal 5/6; T in 1/6	10–25

[a]Agents administered i.v. 40–60 min (except Group XVII) after i.v. tumour inoculation (8×10^6 cells), 10th day of incubation; rub, rubidomycin; Triton, Triton WR 1339; Macro, macrocyclon; BPA, bovine plasma albumin; BGG, bovine gamma globulin.

[b]Most of the deaths occurring during the first day after inoculation/injection ascribed to excessive haemorrhage.

[c]Live embryos only, 15th day of incubation.

[d]T, tumour growth easily visible; HT, heavy tumour growth.

[e]By microscopic examination of liver section (stained H and E); necrotic areas often present when tumour growth substantial.

[f]Ia and IIa were the control groups.

[g]Ib and IIb were the control groups.

[h]Ic and IIc were the control groups.

[i]Id and IId were the control groups.

[j]Delayed treatment; inoculation on day 10; treatment 24 hr later.

[k]Ie and IIe were the the controls groups.

*Table 3. Antitumour effect of combinations of Gem 30 and Gem 15 with rubidomycin and rubomycin**

Group	Dose†/embryo	No. of embryos	Survivals on incubation day 15	Mean wt (g) on day 15 of Embryo(E)	Liver(L)	Mean E/L	Macroscopic appearance of liver
Normal embryos	No treatment	6	6	9·8	0·189	52	Normal
Tumour-bearing embryos	No treatment	11	11	10·7	0·541	20	Heavy tumour growth in 10/11 livers
Rubidomycin + Gem 30	10 μg + 1·5 mg	8	6	12·0	0·269	45	Normal
Rubomycin + Gem 30	20 μg + 1·5 mg	8	8	10·7	0·237	45	Normal
Rubidomycin + Gem 15	10 μg + 0·5 mg	9	8	11·6	0·369	31	No tumour visible in 6/8 livers

*Inoculum of 10×10^6 Landschütz tumour cells/embryo; 10th day of incubation.
†Injected i.v. approx. 40 min after inoculation.

Table 4. Effect of rubomycin and Triton WR 1339 upon Landschütz ascites tumour growth in the chick embryo (inoculation and injection† on the 11th day of incubation)*

Group	Dose/embryo	Survival at 16th day of incubation	Mean liver wt (mg)	Macroscopic or histological examination
Normal embryos	No treatment	10/10	350	—
Tumour-bearing embryos	No treatment	5/10	1500	Massive tumour growth
Rubomycin	50 μg	3/5	842	Tumour cells in liver
Triton	12·5 mg	3/10	1800	Massive tumour growth
Rubomycin + Triton	50 μg + 12·5 mg	3/5	386	Tumour cells in liver

*10×10^6 tumour cells i.v./embryo.
†i.v. approx. 30 min after tumour inoculation.

*Table 5. Suppressive effect of cyclophosphamide upon Landschütz ascites tumour growth in the chick embryo**

Dose† (mg/embryo)	Time‡	Survivors§	Mean liver wt. (mg)	Macroscopic or histological examination of liver
1·0	5 min	1/5	421	No tumour cells
0·5	5 min	3/4	470	
0·25	5 min	4/4	443	Discrete tumour cells
0·125	5 min	5/5	300	
0·02	5 min	4/5	960	Heavy tumour growth
0·25	1 hr	4/4	357‖	Discrete groups of tumour cells
0·25	24 hr	5/6	517‖	
0·25	48 hr	5/6	423‖	
Tumour-bearing controls (no treatment)		3/5	1215	Massive tumour growth
		3/5	1316‖	
Normal embryos (no treatment)		6/6	400	—
		5/5	317‖	

*10×10^6 tumour cells inoculated i.v. into 11-day embryo.
†i.v. ‡After inoculation.
§No. surviving/no. injected (16th day of incubation). ‖Separate experiment.

non-toxic to the 10-day embryos. Potentiation of rubidomycin was also seen with Triton WR 1339 and Poly 30, but they appeared to be less active than Gem 30 even at higher doses. Macrocyclon did not potentiate rubidomycin nor did the relatively toxic Mono 30. None of the detergents given alone showed an antitumour effect and the protein–antibiotic combinations were inactive.

A combination of rubidomycin and Gem 30 administered 24 hr after the inoculation of 10-day embryos was also inhibitory, although the delay led to less effective therapy (Table 2).

Gem 15 was less effective than Gem 30 when given with the antibiotic at about the same fraction of the toxic dose (Table 3).

When embryos were inoculated and treated on the 11th day of incubation, it was possible to administer a dose of antibiotic large enough by itself to affect tumour growth. For example, 50 μg rubomycin was partially inhibitory; its effect was again enhanced (as indicated by liver weight) by Triton WR 1339 (Table 4).

In experiments carried out with Mr. Mervyn Jones, rubidomycin given intraperitoneally at a dose rate of 1·0 mg per kg per day for 4 days, starting the day following intraperitoneal inoculation, increased the survival time of Landschütz ascites tumour-bearing C-male mice by about 70%. Simultaneous administration of the antibiotic with Poly 30

(1·5 g/kg/day) or Gem 15 (50 mg/kg/day) did not significantly improve this figure.

DISCUSSION

The reason for making and including Gem 15 and Gem 30 in the detergent study was an unpublished finding by Mr. I. G. Knight and Dr. R. B. Beechey [12]. They showed that Triton WR 1339 inhibited the ATP-dependent reduction of NAD by succinate in the presence of sub-mitochondrial particles (> 60% inhibition at *ca.* 0·2 μM Triton; *cf.* Devlin and Fabian [13]), but that macrocyclon and a similar macrocyclic structure with longer polyoxyethylene chains had no inhibitory effect. This observation may be correlated with that of Janicki and Miller referred to above, and with the tumour inhibition studies presented here. In each case, activity was shown by the linear polymers but not by macrocyclon. It is possible that this activity is associated specifically with the presence of Gem-type polymers. Rees and D'Arcy Hart [14] showed that such dicyclic structures were exceptional in that they strongly differentiated *in vitro* between the H37Ra (avirulent) and H37Rv (virulent) strains of tubercle bacillus. The mono- or tri-cyclic congeners did not show such a differential effect. Nevertheless, the proposal that the Gem components were primarily responsible for the inhibitory effects seen in the tumour or enzyme studies described above is entirely speculative.

The inactivity of Tween 80 in our experiments could be due to metabolic degradation, although detergency alone is not the only requirement, as shown by the results with macrocyclon.

Albumin and globulin were included in the study because it had been demonstrated earlier in this Institute that the coupling or complexing of certain cytotoxic alkylating agents with proteins sometimes led to an improved therapeutic index against a murine plasma-cell tumour [15].

The experiments with cyclophosphamide confirmed that the chick embryo system, as employed here, can provide a useful *in vivo* tumour screen, and a variant of the chick embryo screening method described by Hellmann and Tucker [16]. It was clear throughout the work, however, that livers having a normal appearance to the naked eye could contain tumour cells in some abundance, so that tumour cure cannot be claimed without supporting histological evidence.

From previous work [8] it is known that within 24 hr of the injection of Landschütz tumour most of the tumour cells have become extravascular although their number has not yet increased significantly. The reduced tumour inhibition obtained with combinations of rubidomycin and Gem 30 injected 24 hr after tumour inoculation may be related to the extravascular situation of most of the tumour cells at this time.

Franchi *et al.* [2] concluded from their study of the ability of Triton WR 1339 to inhibit the formation of metastases in mice implanted with Lewis lung sarcoma, Ehrlich carcinoma and Sarcoma 180, that the antimetastatic effect of Triton was probably related to its capacity to enhance the activity of the reticuloendothelial system and to inhibit movement of the tumour cells. Previous work [8] demonstrated that the failure of a number of murine tumours to grow in chick embryos following intravenous injection was associated with extensive and rapid phagocytosis of tumour cells in the blood vessels. None of the detergents administered alone in the chick embryo system detectably inhibited the establishment of tumour growth in the livers, however, so that the antimetastatic action of Triton by itself, suggested by Franchi *et al.* [2], would not appear to be operating effectively in the chick embryo system. This may be related to the large number of tumour cells (10^7) presented to the host in a single inoculum in this system, whereas Franchi *et al.* were dealing with the much more extended release of tumour cells from tumours implanted subcutaneously, intratibially and intracerebrally. It cannot be excluded that the potentiation of rubidomycin by Triton WR 1339 and by Gem 30 may involve stimulation of phagocytosis of tumour cells which have escaped damage by rubidomycin, particularly as phagocytosis is known to operate very effectively in chick embryos against cells of a variety of tumours introduced into the circulation [8].

D'Arcy Hart and co-workers [17] have proposed that a lysosomal lipid, induced *in vivo* by Triton WR 1339 or macrocyclon, may be responsible for the inhibition of growth of tubercle bacilli in macrophages in animals under detergent treatment. This finding may have a bearing on the chick embryo results described here. An additional possibility is that the uptake of the antibiotics by the tumour cells is increased by detergents of the linear Triton type.

The inability of Gem 15 or Poly 30 to enhance the tumour inhibitory action of

rubidomycin on Landschütz ascites tumours growing intraperitoneally in mice may be related to decreased exposure of the tumour cells in this site to cells of the reticuloendothelial system.

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Tumours of the Peripheral and Central Nervous System Induced in BD-Rats by Prenatal Application of Methyl Methanesulfonate

P. KLEIHUES, CHR. MENDE and W. REUCHER

Max-Planck-Institut für Hirnforschung, Abteilung Allgemeine Neurologie, Köln-Merheim, West Germany

Abstract—*The intravenous injection of methyl methanesulfonate* (20–68 *mg/kg*) *in BD IX rats on the* 15*th and* 21*st day of gestation led to the development of malignant neurogenic tumours in approximately* 20% *of the offspring. Two tumours were located in the cerebral hemispheres (classified as oligodendroglioma and mixed glioma) and six tumours (malignant neurinomas) originated from the peripheral nervous system. The average postnatal survival time of animals with neurogenic tumours was* 292 *days. No neurogenic tumours were found in the offspring of pregnant females treated with methyl methanesulfonate on the* 9*th day of gestation.*

INTRODUCTION

SEVERAL alkylating agents have been reported to induce selectively malignant tumours of the nervous system after systemic application *in vivo*. The most effective neuro-oncogenic compounds known so far are acyl-alkyl-nitrosamides [1], azo- and azoxy-alkanes [2], and aryl-dialkyl-triazenes [3]. There is evidence that these compounds exert their biological effects through an active intermediate which is formed during their metabolism *in vivo*. The ultimate electrophilic structure is assumed to be a carbonium ion which may react with various cell constituents. The alkylation of nucleic acids by the respective methyl and ethyl cation was demonstrated both *in vitro* [4–6], and in the intact animal [7–9] and has tentatively been interpreted as being the relevant reaction for the induction of tumours. It was found, however, that the level of alkylation of nucleic acids in the brain and other rat organs showed no consistent relation to the localization of tumours [7, 8]. Several authors have questioned the significance of the alkylation of nucleic acids [10–12] and argued that other breakdown products may instead be responsible for the carcinogenic action of this group of compounds.

Recent reports are, therefore, of relevance which indicate that direct alkylating agents may also be potent carcinogens both at the site of injection and in distant organs, e.g. uracil mustard [13], propanesultone [14] and methyl methanesulfonate [15, 16]. These compounds are administered in their reactive form and it seems difficult to assume a mechanism of action other than by alkylation of cell constituents.

This report describes the transplacental carcinogenic activity of methyl methanesulfonate in BD IX rats. The intravenous injection in pregnant females on the 15th or 21st day of gestation led to the development of malignant tumours of the peripheral and central nervous system in approximately 20% of the offspring.

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METHODS

Animals. Experiments were started with 12 female BD IX rats [17]. They were mated over-night. When sperm was found on the next

Table 1. *Induction of neurogenic tumours by prenatal application of methyl methanesulfonate. Survey of experimental data*

Day of gestation	Dose (mg/kg)	No. of progeny	Tumour	Localization	Survival time (days)
9	10	20	—		
	20	9	—		
	40	6	—		
15	20	10	Oligodendroglioma	Base of 3rd ventricle	302
			Malignant neurinoma	Lumbosacral plexus	259
	40	5	Malignant neurinoma	Lumbosacral plexus	220
			Mixed glioma	Cerebral hemisphere	
21	40	7	Malignant neurinoma	Lumbosacral plexus	507
	68	10	Malignant neurinoma	Trigeminal nerve	326
			Malignant neurinomas	Lumbosacral plexus (2)	140

morning, this day was designated first day of gestation. Animals were kept on Altromin R diet [18] with water *ad libitum*.

Methyl methanesulfonate was purchased from Eastman Kodak Chemicals, Rochester, N.Y., U.S.A. It was dissolved in sterile saline to a concentration of 10 mg/ml immediately before use.

Injections were given intravenously (V. femoralis) during light ether anaesthesia on the 9th, 15th or 21st day of gestation. The dose was 10, 20, 40 or 68 mg/kg. The number of offspring in the respective dose groups is listed in Table 1. Newborn animals which died within the first month after birth were not considered in the evaluation of data.

Histological examinations. Tissues were fixed in 4% formol saline and embedded in paraplast (trade mark of Shandon). Routine stains were H.E., cresylviolet and reticulin fiber impregnation (Wilder). Samples were taken from the brain and spinal cord and, in most cases, from liver, lungs and kidneys.

RESULTS

Incidence of tumours and survival time

A total of 67 offspring survived the postnatal period. No gross malformations were observed at the doses applied in this study.

Among the 32 offspring of animals treated on the 15th or 21st day of gestation 6 developed malignant tumours of the nervous system (2 brain tumours and 6 tumours of the peripheral nervous system). The average postnatal survival time of rats with neurogenic tumours was 292 days. No neurogenic tumours were found in 35 offspring of pregnant females which were treated with methyl methanesulfonate on the 9th day of gestation (Table 1).

Two animals (585 and 680 days survival time) died from carcinomas of the mammary gland, one from a nephroblastoma (40 mg/kg, 9th day of gestation, 398 days survival time), and another with a subcutaneously located unclassified round cell tumour (648 days survival time). In one rat a pituitary adenoma was found (648 days).

Location and morphology of neurogenic tumours

The classification of neurogenic tumours was based on the morphology of human brain tumours [19] and on previous histopathological analyses of chemically induced neurogenic tumours of the rat [20–23]. Of the two brain tumours observed in this study one was located in the left cerebral hemisphere (basal ganglia). Histopathologically it consisted of neoplastic astrocytes and oligodendrocytes and infiltrated the adjacent brain tissue. It was classified as (malignant) mixed glioma (Fig. 1, upper). In another animal a brain tumour was detected at the base of the third ventricle. This tumour revealed the characteristic histological pattern of an isomorphous oligodendroglioma (Fig. 1, lower) with dark round nuclei surrounded by lightly stained vacuolated spaces (so-called honeycomb structure). The central part of the tumour was somewhat more pleomorphic and showed focal necroses and haemorrhages.

The tumours of the peripheral nervous system originated preferentially from the lumbar-sacral plexus and usually infiltrated both the adjacent muscles (M.psoas) and the spinal cord. Histopathologically, they resembled malignant neurinomas with various degrees of

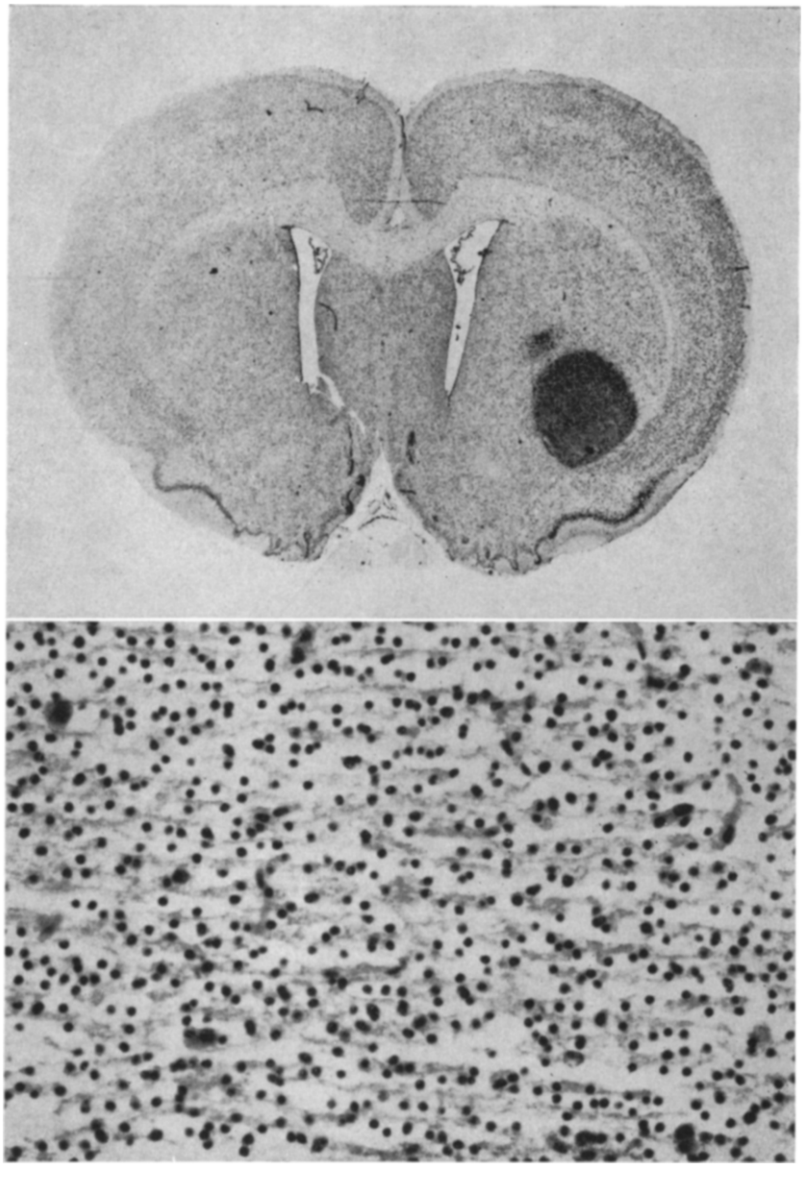

Fig. 1. *Malignant brain tumours induced by prenatal application of methyl methanesulfonate.*

upper: *mixed glioma in the left cerebral hemisphere* (40 *mg/kg*, 15*th day of gestation*, *postnatal survival time* 220 *days*). *Cresylviolet*, 10 ×

lower: *isomorphous oligodendroglioma* (20 *mg/kg*, 15*th day of gestation*, 302 *days*). *Hematoxylin-eosin*, 312 ×

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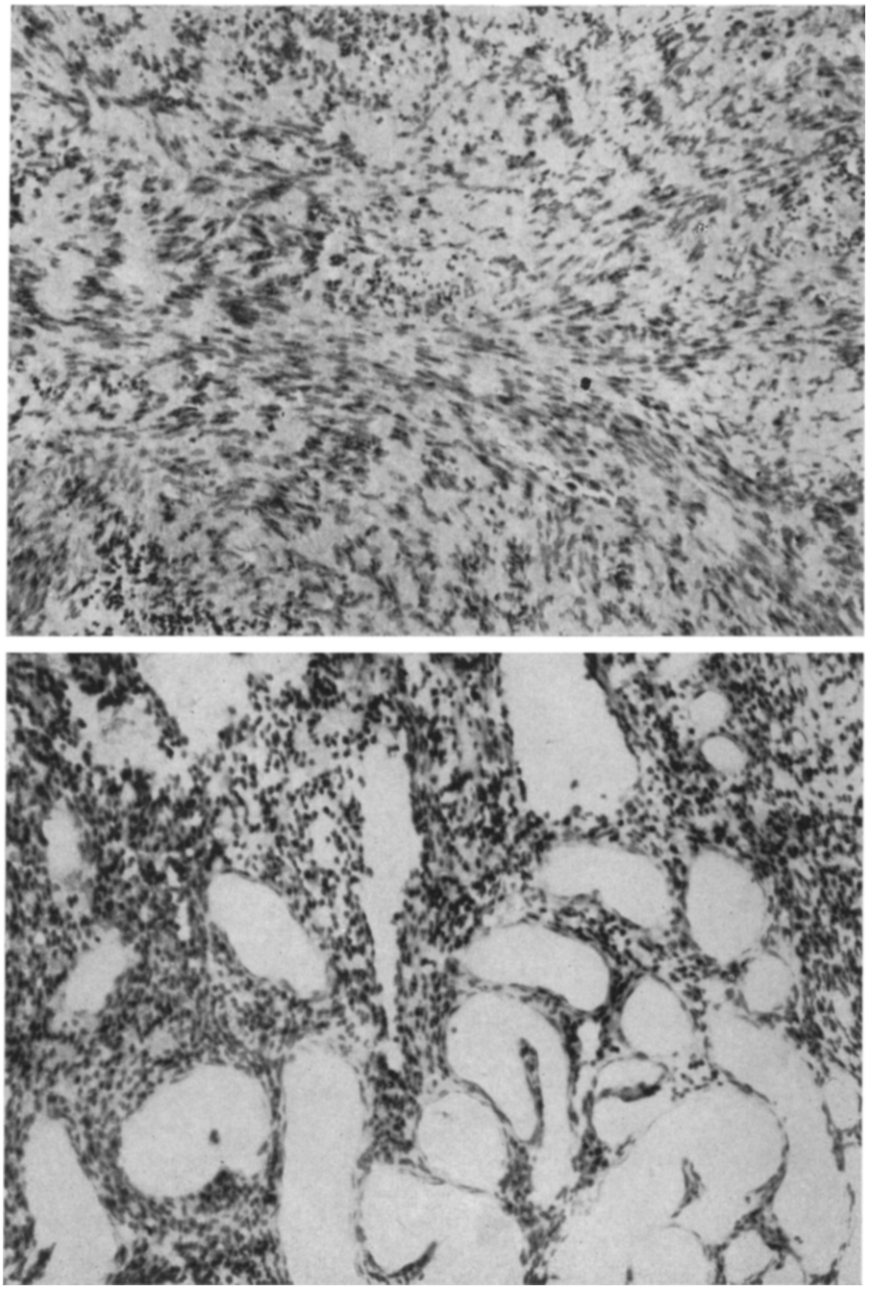

Fig. 2. Malignant neurinomas induced by prenatal application of methyl methanesulfonate.
upper: *differentiated neurinoma of the trigeminal nerve* (68 *mg/kg*, 21*st day of gestation, postnatal survival time* 326 *days*). *Hematoxylin-eosin*, 125×
lower: *cystic neurinoma originating from the lumbar-sacral plexus* (68 *mg/kg*, 21*st day of gestation*, 140 *days*). *Hematoxylin-eosin*, 140×

cellular differentiation. Multiple focal haemorrhages with subsequent cystic degeneration were a common feature (Fig. 2, lower). One rat died from a malignant neurinoma which originated from the proximal portion of the trigeminal nerve. This tumour was well differentiated and showed the characteristic fascicular architecture typical for neurinomas both in man and experimental animals. (Fig. 2, upper).

DISCUSSION

The oncogenic effect of methyl methanesulfonate was first demonstrated in experiments by Clapp *et al.* [24]. When administered to male RF mice in the drinking water (30 mg/kg body weight/day) methyl methanesulfonate significantly increased the incidences of lung adenomas and thymic lymphomas. Prolonged treatment of male rats, on the other hand, did not lead to the induction of tumours. Similarly, Druckrey *et al.* [16] after weekly i.v. injections of 4 or 8 mg/kg (total dose 184 and 368 mg/kg) only observed two skin tumours in a total of 24 BD-rats. But when similar doses were administered subcutaneously (total dose 368 mg/kg), local sarcomas developed in about one-third of experimental animals. Swann and Magee [15] studied the effect of a single (72–120 mg/kg) and of three separate intraperitoneal injections of methyl methanesulfonate in Wistar rats. Of 71 animals 5 developed tumours of the nervous system (3 brain tumours, one tumour of the peripheral nervous system, and one meningeoma). These experiments, therefore, indicated a moderate carcinogenic activity of methyl methanesulfonate in rats, both at the site of injection and in distant organs, with a striking preference for the nervous system.

The results of the present study substantiate the oncogenicity of methyl methanesulfonate in rats and give further evidence for the increased susceptibility of the developing nervous system towards chemical carcinogens [2, 3, 25]. When given as a single dose on the 15th or 21st day of gestation, approximately 20% of the offspring died with malignant neurogenic tumours. A dose as small as 20 mg/kg administered to the pregnant female on the 15th day of gestation was sufficient to induce malignant tumours both in the brain and the peripheral nervous system. As in the case of ethyl-nitrosourea [25], no neurogenic tumours could be induced before the 12th day of gestation and the proportion of tumours originating from the peripheral nervous system was greater than following the application in adult animals [15]. The tumours induced by methyl methansulfonate had identical sites of preferential location (cerebral hemispheres, lumbar-sacral plexus) and were morphologically indistinguishable from tumours induced by neuro-oncogenic nitrosamides.

The tumours observed in organs other than the nervous system cannot be positively related to the injection of methyl methanesulfonate. Their average induction time was twice as long as that of neurogenic tumours and both mammary carcinomas and pituitary adenomas have been observed to develop spontaneously in aged BD-rats [17, 26].

The neuro-oncogenic effect of a direct alkylating agent like methyl methanesulfonate supports the hypothesis that the alkylation of cell constituents can be a sufficient stimulus for the development of malignant tumours. It further indicates that the selective induction of tumours in different organs of the rat (organotropic effect, Druckrey *et al.* [13]) is not restricted to indirect carcinogens like nitrosamines, azo- and azoxy-alkanes, and triazenes.

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Activity of Gastrocnemius and Soleus Polyribosomes in Rats Bearing the Walker 256 Carcinoma*†

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

Department of Biochemistry, Irvine Building, University of St. Andrews, St. Andrews, Fife, Scotland, U.K.

Abstract—*In rats bearing a Walker* 256 *carcinoma the ability of polyribosomal preparations from the gastrocnemius to incorporate* ^{3}H*-lysine into protein under* in vitro *conditions was only* 36% *of the normal level whereas soleus polyribosomes from these animals still retained* 84% *of their normal activity. The alteration in activity of gastrocnemius polyribosomes in tumour-bearing rats was not accompanied by any alteration in the distribution pattern of ribosomal aggregates as evidenced by density gradient sedimentation analysis.*

THE PRESENCE of a growing tumour causes a general wasting of the tissues of the host which is particularly reflected in a loss of muscle protein [1]. In rats bearing the Walker 256 carcinoma both gastrocnemius and soleus muscles exhibit a loss of protein, the gastrocnemius being more severely depleted than the soleus. The *in vivo* incorporation of ^{14}C-valine into the proteins of the gastrocnemius is decreased in the tumour-bearing animal while incorporation into soleus protein is unaffected [2]. These results suggest that some inhibition of protein synthesis may be occurring in the gastrocnemius.

In the present communication, a study has been made of the protein synthetic ability of polyribosomes isolated from gastrocnemius and soleus muscles of rats bearing the Walker 256 carcinoma. The results show that the protein synthetic ability of polysome preparations from gastrocnemius muscles of tumour-bearing rats is consistently reduced below control levels, whereas that of the soleus is not.

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MATERIAL AND METHODS

Animals

Adult female Wistar rats, weighed after an overnight fast, were injected subcutaneously with a suspension of Walker 256 carcinoma cells and paired with control rats on the basis of initial body weight. Rats were housed individually and fed daily 14 g finely ground rat cake (North Eastern Agricultural Co-operative, Ltd.) made into a paste with water, all of which was consumed completely by both groups throughout the experimental period. After 10 to 12 days, when the tumour had grown to about 20 to 30 g, tumour-bearing and control rats were killed and gastrocnemius and soleus muscles removed.

Chemicals and enzymes

Crystalline bovine pancreatic ribonuclease, creatine phosphokinase, trizma base, adenosine triphosphate (ATP), guanosine triphosphate (GTP), creatine phosphate and ribonuclease-free sucrose were purchased from Sigma Chemical Co., London. Lubrol WX was gifted by Imperial Chemical Industries, Ltd., Manchester. Sodium deoxycholate, "Mann Assayed", was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A.,

L-lysine-(4,5)-^{3}H(n).monohydrochloride (250 mC$_i$/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. NE 233 liquid scintillator was obtained from Nuclear Enterprises Ltd., Edinburgh.

Polyribosome preparation

The right and left gastrocnemius from 2 rats and the right and left soleus from 8–16 rats were pooled and homogenised at 0° in medium A [3] with an Ultra-Turrax homogeniser (Janke and Kunkel Kg., Staufen i. Br., W. Germany) running at 80 v for 2 periods of 20 sec. Polyribosomes were isolated from these homogenates by the method of Chen and Young [3] and stored at −40°C overnight. Polyribosome pellets were resuspended in medium A and either used for *in vitro* incorporation studies or sedimentation analysis. Preparations were made simultaneously from muscles of control and tumour-bearing rats.

In vitro *amino acid incorporation*

For the assay of amino acid incorporation, 0·1 ml polyribosome preparation was incubated for 30 min at 37°C in a total volume of 0·5 ml in a medium containing 25 μmole tris-HCl buffer, pH 7·6, 5 μmole $MgCl_2$, 35 μmole KCl, 1 μmole ATP, 0·5 μmole GTP, 10 μg creatine phosphokinase, 7·5 μmole creatine phosphate, 1·67 μC$_i$ ^{3}H-(4,5)-L-lysine and a preparation of liver cell sap [4] from a normal rat, containing approximately 0·6 mg protein. Incubations of polyribosomes from control and tumour-bearing rats were run in parallel and in duplicate and the amount of ribonucleic acid (RNA) in each tube adjusted to the same value, usually 30 to 35 μg RNA. Blank tubes were also incubated containing all constituents except polyribosomes. The reaction was terminated by the addition of 1 ml 10% (w/v) trichloracetic acid (TCA). One half millilitre cell sap preparation (10 mg protein) was added and tubes heated at 90°C for 10 min. Tubes were centrifuged, supernatant discarded and precipitate again extracted with 1 ml 10% (w/v) TCA at 90°C for 10 min. The protein was dissolved in 0·5 ml 0·5 N NaOH and reprecipitated by the addition of 1 ml 10% (w/v) TCA. Tubes were centrifuged and the protein washed 3 times with 5% (w/v) TCA, once with ethanol: ether (1:1) and twice with ether. The protein was dissolved in 0·5 ml 0·3 N NaOH by incubating at 37°C. Digests were diluted with water to give a final alkali concentration of 0·05 N and 0·15 ml aliquots were pipetted onto glass fibre discs (GF/A, 2·5 cm dia) which were dried under infra-red lamps. Three discs prepared from each protein solution were placed in a glass vial with 4 ml NE 233 liquid scintillator and counted on a Beckman LS 100 scintillation counter. Corrections were made for self-absorption due to protein and activity was expressed as disintegrations per minute (dpm) per 100 μg RNA.

RNA content of polyribosomes was estimated from the absorption at 260 mμ of acid-soluble nucleotides resulting from the alkaline digestion of the polysomal suspension. Protein estimations were carried out by the method of Gornall *et al.* [5].

Sucrose-gradient analysis

Polyribosome preparations were layered on a linear 15–40 (w/v)% sucrose gradient in medium A [3], prepared according to the method of Britten and Roberts [6]. The gradient was centrifuged for 1 hr at 39,000 rev/min in the 3 × 5 ml MSE swing-out rotor at 4°C. At the end of the run, tubes were punctured and 21 fractions collected and their absorbancy measured at 260 mμ. The position of the monomer peak was ascertained by incubating a preparation of polyribosomes from a control muscle with 5 μg pancreatic ribonuclease for 15 min at 37°C before placing the preparation on the gradient.

RESULTS AND DISCUSSION

Table 1 compares the incorporation of ^{3}H-lysine into protein by gastrocnemius polysomes isolated from control and tumour-bearing rats. In all five experiments the muscles of the tumour-bearing rats showed a significant weight loss ($P<0·005$). The incorporation of ^{3}H-lysine into protein in the isolated polysomes was greatly reduced in preparations from the tumour-bearing rats, the mean percentage decrease being 64%.

Although the method of preparation of polyribosomes used in these experiments has been claimed to yield a preparation with a relatively low monomer and dimer content, nevertheless the proportion of monomers and dimers in such preparations increases in animals starved for 52 hr [3]. The possibility exists therefore that the low activity of preparations from tumour-bearing rats might be due to a relative increase in monomers and dimers. Figure 1 shows sedimentation profiles from a linear 15 to 40% (w/v) sucrose gradient of polyribosome preparations from gastrocnemius muscles of control and tumour-bearing rats.

Table 1. In vitro *amino acid incorporation by isolated polyribosomes from gastrocnemius muscles of control and tumour-bearing rats.*

Experiment No.	Group	Mean initial body wt. (g)	Mean body wt. change* (g)	Mean tumour wt. (g)	Mean gastrocnemius wt. (g)	Incorporation of ^{3}H-lysine	
						dpm/100 μg RNA	% of control
1†	Control	217	− 7	—	2·65	2370	—
	Tumour-bearing	216	−15	15·7	2·21	1335	56·3
2	Control	220	0	—	2·65	1363	—
	Tumour-bearing	220	−23	24·2	2·20	441	32·4
3	Control	201	0	—	2·44	4978	—
	Tumour-bearing	202	−17	24·0	2·24	2619	52·6
4	Control	199	− 2	—	2·36	4398	—
	Tumour-bearing	197	−14	26·5	1·97	654	14·9
5	Control	178	+ 3	—	2·13	7734	—
	Tumour-bearing	177	0	22·2	2·05	1752	22·7

*Change in weight during experimental period. In the case of the tumour-bearing animals the tumour weight was subtracted from the final body wt.

†Number of rats in each group was 2.

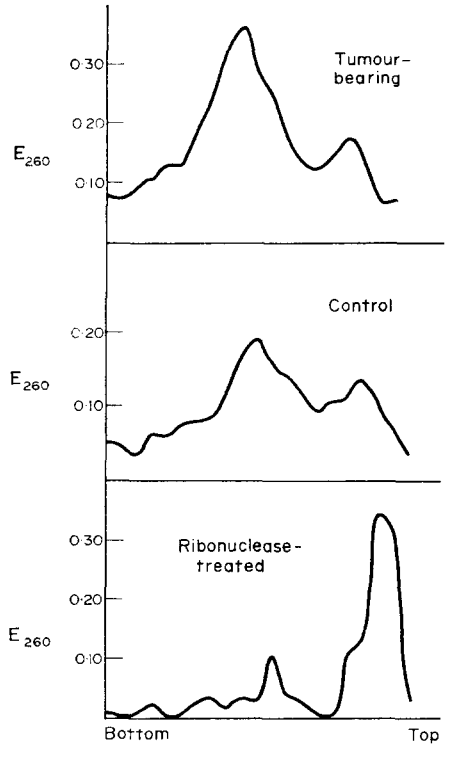

Fig. 1. *Density gradient sedimentation analysis of polyribosomal preparations from gastrocnemius muscles of control and tumour-bearing rats.*

The area representing ribosomal aggregates greater than the dimer was delineated by reference to a ribonuclease-treated polyribosome preparation and the mean percentage distribution calculated.

As can be seen from Table 2, the mean percentage of the gastrocnemius ribosome population present as polyribosomes (>dimer) is $77{\cdot}0 \pm 3{\cdot}9$ in tumour-bearing rats and $70{\cdot}6 \pm 2{\cdot}0$ in control rats. Thus the difference in protein synthetic activity between the two preparations cannot be attributed to an increase in the relative amounts of monomers and dimers in the preparation from the tumour-bearing rats. Other instances have been reported of changes in the rate of *in vitro* amino acid incorporation which cannot readily be explained by concomitant changes in polysomal profile (4, 7–10) and in these cases the existence of regulatory steps in protein synthesis at the polysome level have been proposed.

It was observed previously that in rats bearing a Walker 256 carcinoma, the soleus lost relatively less weight and protein due to the action of the tumour than did the gastrocnemius [2]. In the present work, in contrast to the significant loss in gastrocnemius weight

Table 2. Percentage distribution of ribosomes in gastrocnemius polyribosome preparations from control and tumour-bearing rats

Group	Experiment	Percentage of total population			
		Monomer + dimer	Mean + S.E.M.	Polyribosomes	Mean + S.E.M.
Control	1	33·3		66·7	
	2	27·5	29·4 ± 1·97	72·5	70·6 ± 1·97
	3	27·3		72·7	
Tumour-bearing	1	30·6		69·4	
	2	20·5	23·0 ± 3·58	79·5	77·0 ± 3·9
	3	17·8		82·2	

Table 3. In vitro *amino acid incorporation by isolated polyribosomes from soleus muscles of control and tumour-bearing rats*

Experiment No.	Group	Mean initial body wt. (g)	Mean body wt. change* (g)	Mean tumour wt. (g)	Mean soleus wt. (mg)	Incorporation of ^{3}H-lysine	
						dpm/100 μg RNA	% of Control
1 (8)†	Control	199 ± 6	+ 2	—	175 ± 9	1288	—
	Tumour-bearing	200 ± 6	− 13	24·7 ± 2·1	178 ± 8	1156	89·8
2 (16)	Control	186 ± 4	+ 1	—	181 ± 5	4099	—
	Tumour-bearing	186 ± 8	− 10	26·9 ± 1·9	172 ± 8	2699	65·8
3 (14)	Control	179 ± 4	+ 8	—	176 ± 4	3850	—
	Tumour-bearing	177 ± 4	− 19	28·9 ± 2·2	167 ± 5	3747	97·3

*Change in weight during experimental period. In the case of the tumour-bearing animals the tumour weight was subtracted from the final body wt.

†( ) number of rats in each group.

there was no significant loss in soleus weight in the tumour-bearing animals (Table 3). The tumour was also shown to have no effect on the *in vivo* incorporation of ^{14}C-valine into proteins of the soleus although it caused a significant inhibition of incorporation into the proteins of the gastrocnemius [2]. A similar pattern emerges under *in vitro* conditions in that the incorporation of ^{3}H-lysine into protein by soleus polysomes was inhibited only slightly in tumour-bearing rats, the mean percentage decrease being 15·7% (Table 3). Thus the difference in degree of response of the two muscles to the action of the tumour found in the intact animal is reflected in alterations in the ability of polysomes isolated from these muscles to carry out *in vitro* incorporation of labelled amino acids into protein.

The results obtained in the present work would therefore suggest that the means whereby the tumour causes a depletion of gastrocnemius protein involves an inhibition of the mechanism of protein synthesis at the level of translation on the polysomes.

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Competition Hybridization Studies on Rapidly Sedimenting RNA of Acute Leukemia Blast Cells*

UMBERTO TORELLI, GIUSEPPE TORELLI and CARLO MAURI

Institute of Medical Pathology, University of Modena

Abstract—35S–55S *fractions were obtained by sucrose gradient fractionation of RNA extracted from unlabeled and pulse-labeled normal PHA-stimulated lymphocytes and from acute leukemia blast cells. The latter cells, either unlabeled or incubated for 2 hr with uridine-5-^3H, were "chased" with actinomycin D for 3 hr before extraction. Hybridization competition experiments were performed by preincubating normal lymphocyte DNA with RNA from unlabeled normal PHA-stimulated lymphocytes or leukemic blasts and then challenging the DNA with labeled RNA from normal PHA-stimulated lymphocytes and from acute leukemia blast cells. Unlabeled normal RNA was unable to presaturate most of the sites for labeled leukemic RNA, and unlabeled leukemic RNA was also partially unable to compete with labeled RNA from normal PHA-stimulated lymphocytes.*

These results indicate that leukemic blast cells synthesize metabolically stable heterogeneous nuclear RNA molecules which are not transcribed in PHA stimulated lymphocytes.

WHEN labeled total cell RNA extracted from PHA-stimulated lymphocytes, as well as from HeLa cells, is analyzed by sedimentation in sucrose gradients, the distribution of the radioactivity is dependent on the period of incubation with the labeled precursor. After short incubations (15–30 min) the label is associated mainly with rapidly sedimenting non ribosomal molecules, whereas, after longer periods of contact with the precursor, the radioactivity is mainly associated with the 28S and 18S RNA components of the ribosomal subunits [1–4].

Different behavior has been observed in acute leukemia blast cells. Their macromolecular RNA metabolism is characterized by the association of a major part of the radioactivity with RNA molecules sedimenting in the 35S–55S region of the gradient even after several hours of incubation with the labeled precursor [5, 6].

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RNA components of both the ribosomal precursor and the heterogeneous DNA-like types are spread in the 35S–55S region [1, 7, 8–9].

In rapidly proliferating cells both these classes of RNA molecules are characterized by a high metabolic instability. However, when acute leukemia blast cells are incubated with a labeled RNA precursor, a large portion of radioactivity remains associated with rapidly sedimenting RNA after several hours of actinomycin "chase" [6]. To obtain a better characterization of the RNA with a high molecular weight showing this peculiar metabolic behavior, competition hybridization experiments were performed in order to compare RNA sequences in rapidly sedimenting RNA molecules from normal PHA-stimulated lymphocytes and actinomycin-chased acute leukemia blast cells. The observed lack of cross competition between the two RNA apparently suggests that the leukemic cells are transcribing heterogeneous nuclear RNA molecules which are not transcribed in normal PHA-stimulated lymphocytes.

MATERIAL AND METHODS

Cells and labeling conditions

Leukemic blast cells were obtained from three untreated cases of acute lymphatic leukemia having high levels of circulating leukocytes. Red cells were sedimented by gravity and the supernatant was diluted with autologous plasma and Minimal Essential Medium to obtain a final concentration of 5×10^6 cells per ml with 20% plasma. Previous experiments had shown that such a concentration was not high enough to exhaust any essential amino acid from the medium and to impair protein synthesis in the cultured cells. Actinomycin D was then added to the cultures at the final concentration of 5 μg/ml and the cells harvested 3 hr later. To obtain labeled RNA, uridine-5-^{3}H was added at a final concentration of 20 μc/ml and the cells were incubated for 2 hr and then "chased" for 3 hr with actinomycin D added in the presence of the radioactive precursor.

Normal lymphoblasts were obtained by isolating large amounts of circulating lymphocytes from normal donors by filtering them through nylon filters [6] and maintaining them in culture at the concentration of 3×10^6 cells per ml in the presence of PHA. To obtain rapidly labeled RNA the cells were exposed for 20 min to uridine-5-^{3}H at the concentration of 50 μc/ml and then harvested by cooling rapidly on frozen saline and centrifuging at 1200 rev/min in a refrigerated centrifuge.

Extraction and isolation of RNA

Total cell RNA was extracted by the hot phenol-meta-cresol method already described [6] and precipitated with ethanol. The precipitate was washed twice with cold 3M Na acetate at pH 6 and dissolved in 5 ml of 0·01 M Tris, at pH 7·4. 200 μg of DNAse with 10 μl of 1·0 M Mg acetate were added and the solution was incubated for 10 min at 37°C. Fifty μg of substilisin were then added and the incubation prolonged for 10 min. Half a milliliter of 1·5 M NaCl was then added and the solution extracted with 10 ml of phenol-meta-cresol. The RNA was finally precipitated with 2 volumes of ethanol, dissolved again in 0·01 M Tris buffer at pH 7·4 with 0·1 M NaCl, and centrifuged at 16·000 rev/min in the SW 25·1 rotor of the Beckman L50 ultracentrifuge for 16 hr at 4°C. Several fractions corresponding to S values from 35 to 55 were pooled and precipitated with 2·5 volumes of ethanol, collected by centrifugation and dissolved in 2 × SSC (0·15 M NaCl, 0·015 M Na Citrate) and 0·05 SDS.

DNA preparation

Human DNA was extracted from normal lymphocytes by the Marmur [10] procedure with several deproteinization steps. DNA, dissolved in 0·1 × SSC at a concentration of 100 μg/ml, was denatured by boiling for 5 min and cooled rapidly in ice. It was then filtered through Millipore filters which were dried for 6 hr at 60°C.

Hybridization technique

RNA-DNA hybridization was carried out according to Gillespie and Spiegelman [11]. The filters loaded with DNA were incubated with the appropriate amounts of labeled normal or leukemic RNA for 18 hr at 67°C in scintillation vials in 3 ml of 2 × SSC containing 0·1% SDS. Filters were then drained, soaked 3 times in 20 ml of 2 × SSC for 15 min, and rinsed by suction with 20 ml of the same solution. RNAse digestion was carried out in 2 × SSC for 15 min at 37°C. The filters were finally soaked twice in 20 ml of 2 × SSC, dried at 60°C and counted in a toluene scintillator. Competition experiments were performed by the presaturation technique of Kasai and Bautz [12] as described by Soeiro and Darnell [13]. Filters with DNA were incubated with unlabeled normal or leukemic RNA for 18 hr at 67°C, drained, soaked twice for 30 min in 20 ml of 2 × SSC at 65°C and then returned to vials for further incubation with labeled normal or leukemic RNA.

RESULTS

Figure 1 shows the sedimentation pattern of total cell RNA extracted from normal human lymphoblasts incubated with uridine-5-^{3}H for 20 min. Most of the radioactivity is associated with RNA sedimenting in the gradient fractions corresponding to 35S–55S. These fractions were pooled and the corresponding RNA used in hybridization experiments.

The changes induced in the distribution of RNA radioactivity by actinomycin chase are indicated in Fig. 2 A and B, showing patterns obtained sedimenting labeled total cell RNA extracted from leukemic blast cells of case no. 1 incubated with uridine-5-^{3}H and ^{14}C-methyl-methionine for 2 hr (A) and then chased with actinomycin D for 3 hr (B).

It is apparent that a fairly large amount of the tritiated precursor was left associated, after actinomycin chase, with unmethylated RNA sedimenting in fractions corresponding to 35S–55S. In Fig. 2B are also indicated, as well

as in Fig. 3, the gradient fractions from which the leukemic RNA used in hybridization competition experiments was obtained.

Since the aim of this investigation was to perform competition experiments under conditions such that "saturation" of DNA was obtained, increasing amounts of radioactive

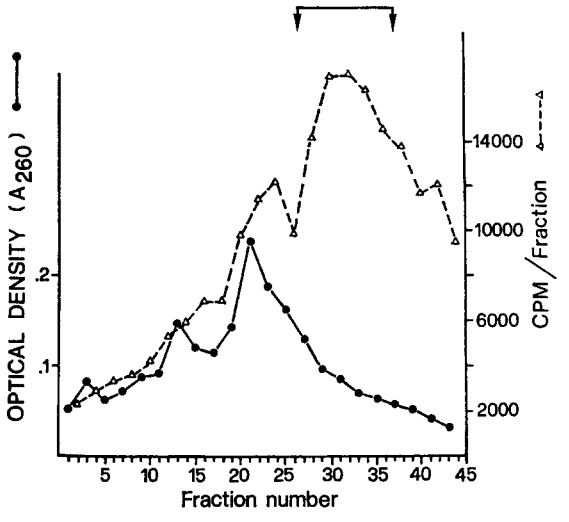


Fig. 1. Sedimentation profile of radioactive RNA extracted from PHA-stimulated lymphocytes incubated for 20 min with ^{3}H-uridine. Centrifugation for 16 hr at 16,000 rev/min on 5 to 20% sucrose gradients. Sedimentation from left to right. The fractions indicated by arrows were pooled from several gradients and used in hybridization experiments. Unlabeled RNA extracted from PHA-stimulated lymphocytes was centrifuged at the same speed and for the same period of time on several gradients. The fractions indicated by arrows were pooled and used to presaturate the DNA in competition hybridization experiments.

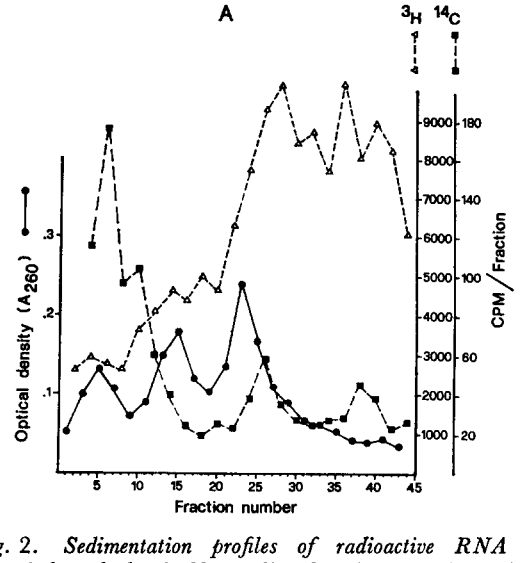
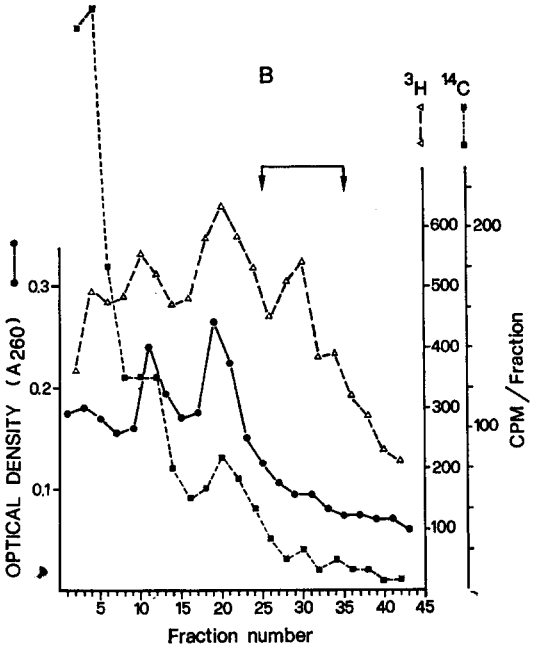


Fig. 2. Sedimentation profiles of radioactive RNA extracted from leukemic blast cells of patient no. 1 incubated with ^{3}H-uridine (20 μc/ml) and ^{14}C-methyl-methionine (5 μc/ml). A: RNA extracted from cells incubated for 2 hr with the labeled precursors. Centrifugation at 16,000 rev/min for 16 hr on 5 to 20% sucrose gradients.



B: RNA extracted from cells incubated for 2 hr with the labeled precursors and then chased for 3 hr with actinomycin D.

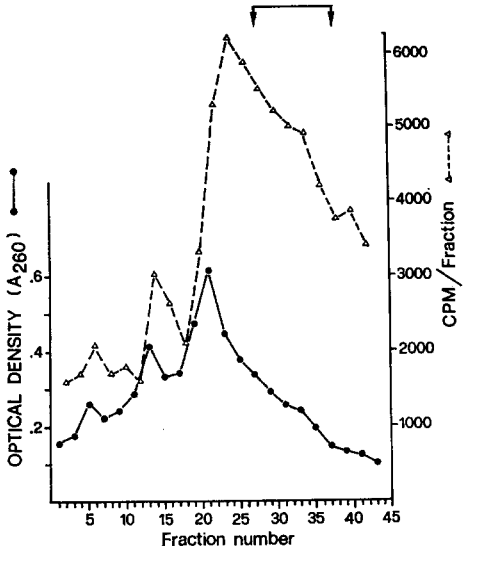


Fig. 3. Sedimentation profile of radioactive RNA extracted from leukemic blast cells of patient no. 2 incubated with ^{3}H-uridine for 2 hr and then chased for 3 hr with actinomycin D. Centrifugation at 16,000 rev/min for 16 hr. Fractions indicated by arrows were pooled from several gradients and used in hybridization experiments.

RNA were hybridized to filters containing 5 μg of human DNA. Normal RNA with a specific activity of 2000 to 4000 cpm/μg was used in our experiments. The specific activity of leukemic RNA used was much lower (200 to 400 cpm/μg). Representative saturation curves are shown in Fig. 4. The fraction of the genome hybridized at saturation by leukemic RNA was always much lower than that hybridized by normal RNA. Figure 5 shows the

results of competition experiments in which normal lymphocyte DNA was preincubated with increasing amounts of unlabeled normal RNA and then challenged with labeled rapidly sedimenting RNA from both normal PHA-stimulated lymphocytes and leukemic blast

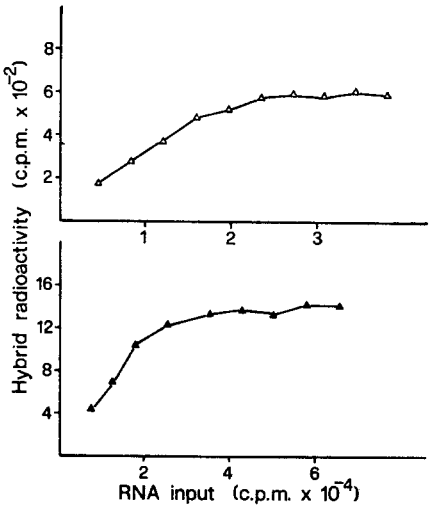


Fig. 4. Saturation of normal lymphocyte DNA by rapidly sedimenting labeled RNA from normal PHA-stimulated lymphocytes (lower panel) and leukemic blast cells (upper panel). Increasing amounts of labeled RNA were hybridized to 5 μg of DNA and the amount of hybrid formed was determined. The reaction was carried out at 67°C for 18 hr. The background on filters containing no DNA was about 15 cpm. The specific activity of normal RNA was 3850 cpm/μg and that of leukemic RNA was 360 cpm/μg.

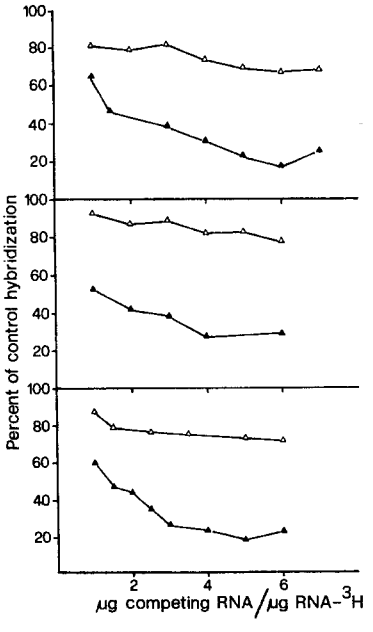


Fig. 5. Effect of increasing amounts of unlabeled rapidly sedimenting RNA from normal PHA stimulated lymphocytes on the hybridization of radioactive RNA from PHA-stimulated lymphocytes (▲———▲) and from leukemic blast cells (△———△). Panels from top to bottom correspond to RNA from patients no. 1, 2 and 3.

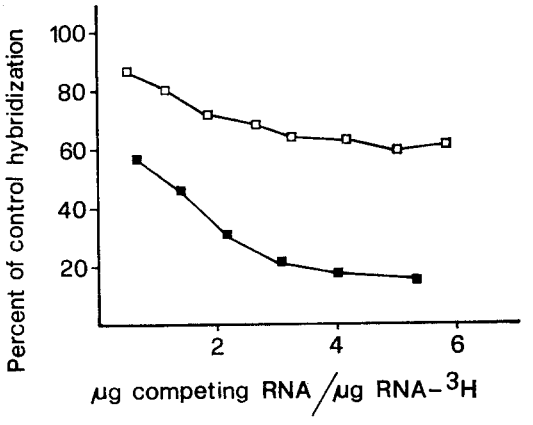


Fig. 6. Effect of increasing amounts of unlabeled, rapidly sedimenting RNA from leukemic blast cells on the hybridization of radioactive RNA from normal PHA-stimulated lymphocytes (□———□) and from leukemic blast cells of patient no. 3 (■———■).

cells. It is apparent that unlabeled normal RNA was unable to presaturate most of the sites for labeled leukemic RNA, whereas hybridization of labeled normal RNA was almost completely inhibited. As shown in Fig. 6, incomplete inhibition was also observed when DNA preincubated with unlabeled leukemic RNA was challenged with labeled RNA from normal PHA-stimulated lymphocytes.

DISCUSSION

The observation of several peculiarities in macromolecular metabolism of ribosomal RNA precursor of leukemic blast cells, such as the marked delay in processing of 32S to 28S RNA and the abortive cleavage of 18S RNA, previously led to the conclusion that the labeled, metabolically stable 35S–55S molecules extracted from leukemic blast cells are mainly formed by 45S ribosomal precursor RNA which does not undergo proper methylation for cleavage, and accumulate [5, 6]. However, the inability of unlabeled 35S–55S RNA from normal PHA-stimulated lymphocytes to compete with labeled 35S–55S RNA from leukemic cells suggests that in these cells the amount of unmethylated unprocessed ribosomal precursor is small.

The labeled 35S–55S RNA found in leukemic cells after actinomycin chase appears to be formed mainly by molecules belonging to the heterogeneous class. The lack of competition between this RNA and the corresponding normal fractions suggests, on the other hand, that leukemic lymphoblasts are transcribing RNA sequences which are repressed in normal PHA-stimulated lymphocytes. However, the

characteristics of the RNA–DNA hybridization reaction must be carefully evaluated. In fact, the formation of RNA–DNA hybrids does not require absolute complementarity of base sequences. Hybridization competition experiments appear mainly useful to discriminate among RNA molecules transcribed from unrelated or different families of reiterated RNA sequences [14, 15]. Under these conditions the observed lack of competition may reflect a ratio of competing RNA to labeled RNA too low to achieve presaturation of repeated sequences correlated to those sequences from which the RNA molecules in leukemic blast cells were transcribed.

No definite conclusion can be reached about the problem whether the differences detected in this study reflect malignancy or rather lack of differentiation. Complementary nuclear RNA molecules undetected in the corresponding normal cells have already been detected in two malignant conditions, human chronic lymphocytic leukemia and transplantable breast cancer of mice and rats [16, 17]. In these studies the authors showed that the RNA from the malignant cells could compete fully with labeled RNA from normal control cells, thus giving support to the interpretation that the pathological cells contained malignancy specific RNA sequences. In our study, unlabeled RNA from malignant cells was unable to compete fully with labeled RNA from normal cells. However, under the conditions of our experiments, this might be explained by the absence of ribosomal precursor in the competing unlabeled RNA.

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Resistance to Methylene Dimethane Sulphonate (MDMS) in L1210 Cells *In Vitro*

DIANA ANDERSON and A. H. W. NIAS*

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, Great Britain

Abstract—*Attempts were made* in vitro *to induce resistance in L1210 cells to the alkylating agent MDMS. Progressively increasing sequential treatments of drug produced a stable resistance which could not be increased by further treatment. This was manifest in the form of a shoulder difference between the dose response curves of the untreated control and resistant populations. A cell line cloned from the resistant line showed a smaller degree of resistance. Large single doses of drug failed to produce resistance, so did sequential treatments when an initial high dose of drug was given. Sequential treatments to a cloned population also did not produce resistance. When the resistant cells were transplanted into mice and assayed* in vivo, *resistance was only evident in the initial tests and reversion to control sensitivity soon occurred. Labelling studies showed no difference between the growth kinetics of the resistant and control populations.*

INTRODUCTION

ONE OF the problems involved in obtaining chemotherapeutic cures in tumour systems is the presence of spontaneous or drug-induced cell variants which may overlap the range of drug sensitivity of vital normal cell populations of the host so preventing a selective killing of the tumour cells. The study of drug resistance in tumour cells is thus a pressing practical problem. Methylene dimethane sulphonate (MDMS) is an effective anti-tumour agent with respect to the Yoshida sarcoma in rats, where cures of 25-g tumours have been obtained after single doses of 10 mg/kg (LD50 = 25 mg/kg) of the drug [1]. Resistance developed in 10% of the treated animals, however, after this single treatment. This posed a question as to whether resistance to this drug develops as readily in other tumour systems. L1210 leukaemia was chosen because it is widely used in chemotherapy studies.

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*Present address: Glasgow Institute of Radiotherapeutics, Belvidere Hospital, Glasgow G31 4PG, Great Britain.

Although a single treatment of methylene dimethane sulphonate *in vivo* produced a resistant Yoshida sarcoma line and this resistance was stable on transference *in vitro* [2], other workers have found that the induction of resistance does not occur so readily to other alkylating agents, particularly *in vitro*. Goldenberg [3] demonstrated that sequential treatments to L5178Y lymphoblasts *in vitro*, with increasing doses of HN_2, eventually resulted in an outgrowth of a less sensitive population but an earlier attempt [4] failed, presumably because the course of treatment was not continued for a sufficient length of time. These workers did not consider single large treatments. In this present investigation, both single and sequential treatments were used *in vitro*. In earlier studies [5] both single and sequential treatments *in vivo* failed to produce resistance.

MATERIAL AND METHODS

Cell line

The L1210 mouse leukaemia was obtained from Dr. B. C. V. Mitchley of the Chester Beatty Research Institute in 1968.

In vitro *culture methods*

L1210 cells were cultured in suspension using techniques which have been published previously for P388F cells [6]. For growth curves the cell cultures were diluted every 2 to 3 days for a 10-day period to maintain exponential growth. For colony counting in dose response studies they were plated into soft agar using the methods described by Nias and Fox [7]. The plating efficiency was 95%. At drug concentrations below 70 μg/ml, 10^3 cells were plated per dish. At higher concentrations, higher cell numbers were used. It was shown that there was a linear relationship between the number of cells plated (10^3, 10^4 and 10^5) at doses of MDMS of 70, 80, 100, 110, 120 and 130 μg/ml. In order to obtain sufficient cells in exponential growth for these plating procedures cell numbers were concentrated. In drug resistance studies cells were treated in exponential growth at a high cell density (1 to 2×10^5 cells/ml.)

Drug

Methylene dimethane sulphonate (MDMS) was obtained from Dr. B. W. Fox in this laboratory. The drug was dissolved in physiological saline for *in vivo* experiments and in Fischer's medium for *in vitro* experiments when it was sterilised by filtration (Millipore 0·22 μ). The dissolved drug was kept on ice to reduce the rate of hydrolysis to a minimum. (Half-life of MDMS at 37°C = 22 min, and at 0°C = several hours). For dose response and resistance studies, cell cultures were exposed to the drug for 15 min at 37°C. After each treatment, drug containing medium was removed by dilution or in the case of high doses of drug and high cell numbers by centrifugation for 10 min (at 800 rev/min) and the cell pellet resuspended in fresh Fischer's medium + 10% Horse Serum.

Labelling studies

Tritiated thymidine (Radiochemical Centre, Amersham) 0·1 μCi/ml and of specific activity 26·6 Ci/mM was used. It was dissolved in Fischer's medium. In labelled mitoses experiments cells were pulse labelled for 30 min. The slides were prepared for autoradiography as described previously [6]. They were exposed for 7 days before development. Labelled mitoses were plotted as a function of time. The common curve was constructed with the arbitrary line of best fit.

RESULTS

In vitro

To induce drug resistance. A dose response curve was determined for the control population of L1210 cells when treated with MDMS. This is shown in the left hand curve in Fig. 1 indicating a D_0 of 7·5 μg/ml and extrapolation number $N = 10$. The fact that a plateau was apparent at a surviving fraction of 2×10^{-4}, after which the survival curve resumed the original slope, suggested the possibility of the presence of a resistant fraction in the population. To investigate this possibility, clones were isolated after treatment with 100 and 130 μg/ml and compared with the untreated control population. These clones, however, showed the same dose response as the control population. Single large doses of 130 μg/ml and 150 μg/ml (Fig. 2A) administered to mass cultures at a cell density of 2×10^5/ml, also failed to confirm a pre-existent resistant subpopulation of cells.

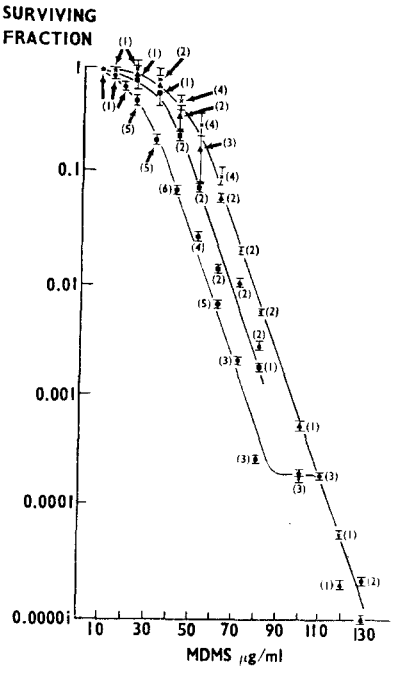


Fig. 1. *Dose response curves for* L1210 *cells when treated with MDMS* in vitro.

● = *untreated control* L1210 *population*

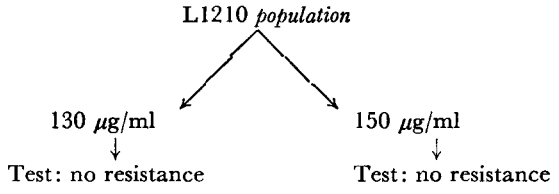
× = *resistant line after an accumulated dose of* 170 μg/ml *MDMS* (*PR* 170).

▲ = *resistant line after an accumulated dose of* 400 μg/ml *MDMS* (*PR* 400).

■ = *clone isolated after treating the line which had accumulated a total dose of* 400 μg/ml *to a further* 60 μg/ml (*CPR* 400 + 60).

The figures in brackets represent the number of different experimental observations per point. Each experiment had a mean of 5 *plates and bars represent their standard errors.*

A. *Single treatment programme of* L1210 *cells with MDMS*



B. *Sequential treatment programme of* L1210 *cells with increasing concentrations of MDMS*

L1210 *population*
↓
20 μg/ml
(14 doublings) ↓
40 μg/ml → Test: no resistance
(18 doublings) ↓
40 μg/ml
(4 doublings) ↓
70 μg/ml → Test: resistant (PR 170)
(4 doublings) ↓
100 μg/ml
(6 doublings) ↓
130 μg/ml → Test: resistant (PR 400)
\+ → 60 μg/ml → clone → Test:
decreased resistance (CPR 400 + 60)

C.

L1210 *clone*
↓
30 μg/ml → Test: no resistance
(20 doublings) ↓
35 μg/ml
(12 doublings) ↓
70 μg/ml
(14 doublings) ↓
100 μg/ml
(16 doublings) ↓
130 μg/ml → Test: no resistance

D.

L1210 *population*
↓
130 μg/ml → Test: no resistance
(24 doublings) ↓
60 μg/ml → Test: no resistance
(32 doublings) ↓
60 μg/ml
(30 doublings) ↓
60 μg/ml → Test: no resistance

Fig. 2. *MDMS treatment schedules for* L1210 *cells.*

When a population of L1210 cells was subjected to the sequential treatments of MDMS, as shown in Fig. 2B, and samples of the population were tested after varying exposures, some resistance was acquired. This was evident after a total accumulated dose of 170 μg/ml (PR 170). When this resistant population was tested against the control population (Fig. 1) the two dose response curves revealed only a shoulder difference, i.e. the extrapolation number was 150 compared with $N = 10$ for the control curve. Further exposures giving a total accumulated dose of 400 μg/ml (PR 400) did not result in an increase in resistance (i.e. N also = 150). Thus, in both cases, the resistant cells showed a 15-fold increase in survival over that of the control cells for the same dose level. This resistance was stable for at least 4 months in continuous culture and was unchanged after the cells had been stored frozen in liquid

nitrogen for a further 4 months and then thawed and retested. The resistant lines showed no plateau in their dose response curve in the range investigated. A cloned line (CPR 400 + 60) isolated after further treating the 400 μg/ml line with 60 μg/ml decreased the original resistance and was intermediate in sensitivity between control and resistant populations ($N = 40$). The D_0 value for the control and resistant populations and clones was the same throughout, i.e. 7·5 μg/ml.

The production of resistance was, however, not repeated by a similar sequential treatment of a different but cloned population (Fig. 2C), nor was it produced with sequential treatments to a population with an initial treatment of a very high dose (130 μg/ml) followed by further moderate treatments (60 μg/ml) (Fig. 2D).

Throughout the sequential treatment programme only healthy exponentially growing cultures were treated. The treatments appear somewhat irregular but they were governed by the "appearance" of the cells, i.e. their morphological integrity and normal growth rate. It was found that the cells would die if they were treated too soon after the previous treatment, i.e. for days or weeks after treatment with the drug (depending on the dose) they were not only unhealthy but were highly sensitive to the drug.

To induce drug sensitivity. In order to obtain two lines with greater differential sensitivity, an attempt was made to isolate stable clones (as opposed to the temporary sensitivity described above) which were even more sensitive than the control population. Uncloned populations were treated with 40 μg/ml of MDMS. After treatment, small clones (i.e. slowly growing) were isolated, 11 from a treated population, 7 from an untreated control population. After isolation, cells from each small clone were grown as a separate culture. Growth curves of these 18 cloned cultures were compared with growth curves of uncloned populations. In spite of the original slow growth of these colonies, there was no significant difference between the growth rates observed. Dose response curves of 3 treated clones and an untreated clone were compared with those of the uncloned population but the "originally small" clones showed no increased sensitivity.

Kinetic investigations of control and resistant populations

Resistance may be conferred by alterations in cell kinetics. However, it was shown by growth curves that the doubling times of the control population and the two resistant populations (PR 170 and PR 400) were all the same, i.e. 12·7 hr. Continuous labelling studies showed no differences between the two cell lines. Similarly, labelled mitoses curves revealed no differences in the cell cycle times or in the distribution of cells between the different phases of the cell cycle (Fig. 3).

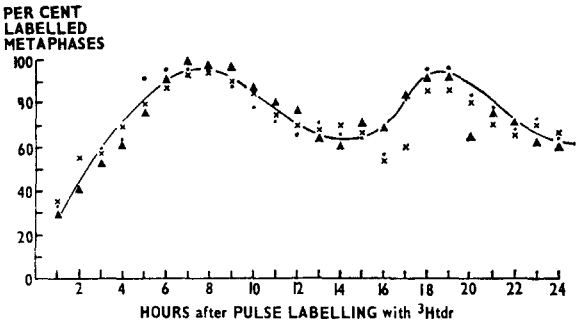


Fig. 3. Labelled mitoses curve
● = *untreated control population*
× = *PR* 170
▲ = *PR* 400

In vitro → in vivo

Both control and resistant cells obtained during culture *in vitro* after an accumulated drug dose of 400 μg/ml (PR 400, Fig. 2B) were inoculated into DBA_2 mice. They were tested through the spleen colony (L1210-CFU) assay system [8, 9] as described earlier [5]. These curves showed a steeper D_0 slope than the *in vitro* curves, and resistance was evident again as a shoulder difference between the control ($D_0 = 3{\cdot}25$ mg/kg; $N = 2{\cdot}4$) and resistant ($D_0 = 3{\cdot}25$ mg/kg; $N = 9{\cdot}0$) dose response curves. This resistance, however, was not stable, and reversion to control sensitivity was seen after 4 weeks passage as an ascites tumour.

DISCUSSION

Attempts were made to produce resistant lines of L1210 leukaemic cells. *In vivo*, no such resistance was produced but, by chance, one resistant line was produced *in vitro*. This resistance was not reproducible and in dose response studies it was evident only as a shoulder difference of control and resistant populations. The resistance could not be increased further. In fact, it decreased when much more treatment was given. The plateau on the dose–response curve of the control population does not appear to be attributable to a pre-existent resistant population since clones isolated after plateau doses show the same response as the control population. A break or plateau in the dose response curve with cytotoxic death is usually

indicative of the presence of a resistant cell population or subpopulation (e.g. resistant in a phase of the cell cycle). In this case, the plateau was at a low level, i.e. 2×10^{-4}. If this were such a resistant phase, it would have to be one lasting only $2 \times 10^{-4} \times 12{\cdot}7$ hr $= 0{\cdot}152$ min.

The resistant line maintained its resistance *in vitro*. On transference of control and resistant lines *in vivo* the resistance was present initially but the degree of resistance was lessened and both lines became far more sensitive (equating mg/kg with μg/ml). This was rather unexpected considering the presumably shorter half-life of MDMS *in vivo*. Resistance, however, was soon lost. This phenomenon has been reported by other workers with other cell lines. Sakurai *et al.* [10] and Goldenberg [3] reported the initial loss of large degrees of resistance from cell populations which then stabilize at a much lower level. Since the degree of resistance here was relatively small, it is not surprising that it was lost completely.

Increased sensitivity to the drug was not produced after isolation of small clones. It has been shown [11–16] that small clones have a slower growth and increased sensitivity. The present results, however, are more in agreement with the results of Wilkinson and Nias [17] who showed different responses (ranging from sensitive to resistant) to nitrogen mustard in different clones selected from a heterogeneous population of HeLa cells.

The kinetic data eliminate the possibility of resistance being conferred by an alteration in the cell cycle. Young and de Vita [18] found that L1210 cells resistant to BCNU (-1,3-bis (2-chloroethyl)-1-nitrosourea) also showed no change in their cell cycle parameters. Other workers, however, have reported kinetic differences for resistant and sensitive cell lines, e.g. Ball *et al.* [19] found the mean length of the G_2 phase longer in the sensitive line than in the Melphalan resistant Yoshida tumour, and Fox and Fox [2] reported an S phase which was almost twice as long in the MDMS resistant Yoshida cell culture. In both cases the kinetic alteration was not considered to be the primary cause of resistance.

The nature of resistance remains unknown. Having excluded the kinetic mechanism, there are a number of other factors to which drug resistance is attributable, e.g. a lower penetration of the drug into or a higher repair capacity of some of the cells or the various other factors discussed by previous authors [2, 3, 5].

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Effects of Single Doses of Radiation on Lung Metastases in Man and Experimental Animals

H. A. VAN PEPERZEEL
Dept. of Radiotherapy, Netherlands Cancer Institute, Amsterdam, The Netherlands

Abstract—*Effects of single doses of radiation were studied on lung metastases of tumours in men, dogs and mice. A correlation was found between growth rate and radiosensitivity as determined from volume reduction of the metastases. In all cases an accelerated growth was observed after reduction of the volume by the irradiation. In this period of accelerated growth in the mouse tumour, an increase in the proliferative fraction and a shortening of the inter-mitotic time of the proliferative cells were measured. Based on the assumption that in the period of accelerated growth also in human tumours there could be a greater radiosensitivity than before irradiation, in two patients the effect of subsequent doses given during the period of accelerated growth, was studied. The results suggest that the second and perhaps the third dose has a greater effect on the volume reduction than the first dose of radiation.*

INTRODUCTION

THE AIM of the present investigations is to compare responses of tumours in animals and men with respect to tumour growth delay and cell proliferation kinetics. Various experimental techniques have been developed by radiobiologists to analyse quantitatively the influence of irradiation on mammalian cell populations.

The method described originally by Puck and Marcus allows studies of the influence of irradiation on the proliferative capacity of cultured mammalian cells. The effect of the irradiation can be expressed by the percentage of cells which after the irradiation are still capable of unlimited reproduction. The results of such experiments can be presented in the well-known survival curves. It is also possible, using this cloning technique, to determine, of an *in vivo* irradiated solid tumour in a laboratory animal, the percentage of tumour cells which have retained the capacity to form a clone *in vitro* [1].

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It is not certain that all the cells in a tumour, which after irradiation form clones *in vitro*, should also have been involved in unlimited proliferation *in vivo*. It is well known that directly after the plating *in vitro* a rapid growth of the cells will take place, but in a later stage of the culture period growth may diminish or stop, even under optimal culture conditions.

Because in this plating procedure cells produce clones containing of the order of 10^3 cells within about 10 days, it must be concluded that their proliferation rate is very high. It is clear that the cells in these *in vitro* conditions behave differently from cells in the clinical situation.

In the case of human tumours, effects of fractionated irradiations have been studied on pulmonary metastases and the results of these investigations [2] indicated the existence of a correlation between the tumour growth rate and the extent of the volume reduction per unit dose, which was used as a measure of the radiosensitivity of the tumour. The lung metastasis model provides an interesting system for the following reasons [3].

1. Every metastasis probably originates from one or a few tumour cells and represents a fairly pure cellular strain. Consequently, each metastasis can be compared to a clone such as is formed in cell culture.
2. Pulmonary metastases of histologically different tumours can be compared with one another with respect to growth rate and radiosensitivity.
3. The slight mechanical resistance offered by the spongelike lung tissue will exert a minimal influence on the growth of metastases.
4. Some metastases in the lung tend to assume a spherical shape and to retain this shape during growth, providing the possibility of reproducible and accurable measurements of their volume in radiographs.
5. Most pulmonary metastases are well vascularized, which manifests itself by a small proportion of necrosis in the metastases. Furthermore cells killed by the radiation are eliminated relatively rapidly as can be deduced from the fact that the decrease of volume can already be measured one week after the first day of the irradiation course. One might assume that the influence of the presence of anoxic cells would not be large.

The difference in density between solid metastases and the spongy lung tissue renders it possible to obtain good contrast on the radiographs, so that the volume growth and reduction can readily be studied.

A disadvantage of the lung metastasis model might be that growth rate and radiosensitivity of metastases are not necessarily equal to those of the primary tumour. In many primary tumours the supply of oxygen and nutrient substances varies considerably from one site to another, while the lack of homogeneity of the surrounding tissues may distort tumour growth. The primary tumour may also consist of parts, which vary in grade of differentiation, and therefore the primary can have a more complicated response to radiation than the metastases.

For a comparison of effects of fractionated irradiation in laboratory animals with those of fractionated irradiation in men, various factors must be considered. Daily fractionated irradiation of rapidly-growing mouse tumours, with a mean inter-mitotic time of the proliferative cells of about 12 hr, implies a treatment with one fraction administered in two cell cycle times. By contrast, daily fractionation in the case of a human tumour with a mean inter-mitotic time of the proliferative cells of about 48 hr, implies the administration of two doses per cell cycle. Effects such as mitotic delay caused by irradiation and the influence of partial synchronization of cells after irradiation can have, in both systems, a totally different influence.

Therefore we decided to investigate also in men the effect of irradiation with single doses of a size that has often been used in clinical practice.

MATERIAL AND METHODS

A. *Effects of irradiation on metastases in patients*

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, data of 11 patients, including 3 women, could be analyzed. Their ages ranged from 25 to 83 years. Two of the patients are still alive, the others died between 3 and 14 months after the termination of the investigation. The histological diagnoses, made by the pathologists of the Academisch Ziekenhuis Wilhelmina Gasthuis and the Antoni van Leeuwenhoek-Huis, are summarized in Table 1.

Table 1.

	Patients	Sex	Age (years)	Histological diagnosis	Measured volume doubling time (days)
(1)	M.S.v.d.B.	♀	35	Rhabdomyosarcoma of the long dorsal muscles	17
(2)	C.S.	♂	29	Embryonal carcinoma of the testicle	21
(3)	J.K.	♂	25	Embryonal carcinoma of the testicle	30
(4)	B.B.	♂	49	Squamous cell carcinoma of the bronchus	39
(5)	A.G.	♂	47	Poorly differentiated adenocarcinoma of the bronchus	57
(6)	W.v.d.W.-d.W.	♀	83	Liposarcoma of the thigh	66
(7)	A.G.S.	♀	42	Fibrosarcoma of the thigh	82
(8)	G.v.d.H.	♂	61	Squamous cell carcinoma of the bronchus	184
(9)	A.T.	♂	44	Anaplastic teratoma of the testicle	185
(10)	M.S.	♂	75	Unknown primary tumour	191
(11)	G.v.B.	♂	67	Anaplastic carcinoma of the bronchus	228

Before the single-dose irradiation was applied, the doubling times of the metastases were determined as described by Breur [4]. These determinations required at least three radiographs of the chest in the posterior–anterior projection. Usually many more radiographs were available. The diameter of the metastases was measured on the successive radiographs. From the circular curcumference of the metastases on the radiographs the diameter of the spherical focus in the lung can be derived. Growth curves presenting the diameter of the metastases as a function of time were drawn on semi-logarithmic paper as shown in Figs. 1 and 2. It was found that in all cases the experimental points are close to straight line, i.e. all 11 tumours examined showed exponential growth, the doubling times ranging from 17 to 228 days (Table 1).

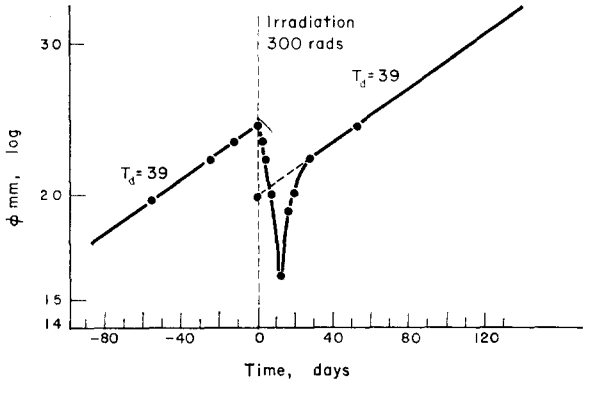

Fig. 1. *Effect of a single-dose of radiation on the growth of a lung metastasis; example of accelerated growth (patient* 4, *Table* 1).

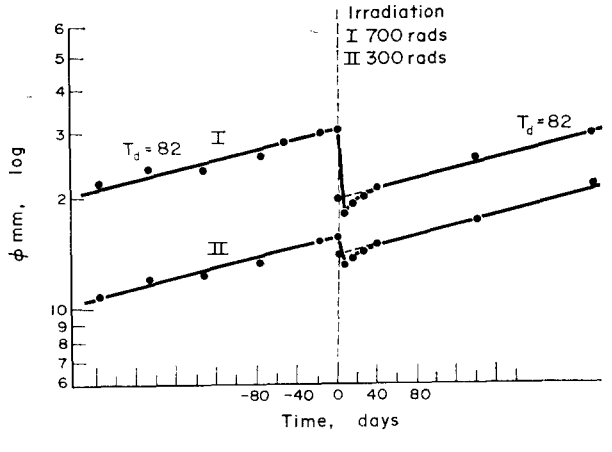

Fig. 2. *Influence of two different doses of radiation on the growth of lung metastases (patient* 7, *Table* 1).

Once the doubling time of the metastases had been determined, one or more metastases were selected for irradiation. Fields were marked such that the dose to the metastases could precisely be calculated. When metastases were irradiated with separate fields, the fields were selected in such a way that the scattered radiation due to the treatment of one metastasis did not contribute a significant dose to the other metastases. The 250 kV X-rays administered had a HVL equal to 3 mm Cu. The dose was always administered through two opposing fields and the dose in the midline was taken to be equal to the tumour dose.

It was expected that after a single-session irradiation the volume of the tumour should decrease. Therefore doses in excess of 700 rads were not applied because too large a dose could result in such a large volume reduction that the accurate measurement of the tumour rest on the radiograph would be no longer feasible and the early stages of regrowth might have been missed. This might occur if the diameter of the metastasis decreases to a value of less than 8 mm, at which size the shadow can no longer be distinguished from that of a tangentially hit blood vessel. After the administration of the selected X-ray dose, many radiographs of the chest were made in the first three weeks after irradiation.

In some patients it was possible to irradiate two groups of metastases with different doses, so that two values of the volume reduction of the metastases could be calculated.

In comparison with cell survival curves, we can determine "volume reduction curves". In these cases we calculated a $D_{0\ \text{volume reduction}}$ defined as that dose which causes the tumour to regrow in a way as if 37% of the tumour volume was left capable of unlimited growth. In most patients it was not possible to apply two different doses of radiation because owing to the site of the different metastases no two irradiation fields could be selected in such a way that the metastases did not receive a significant dose of radiation of the alternative beam. In those cases only one point of the "volume reduction curve" could be calculated and a $D_{0\ \text{vol. red.}}$ can be determined only assuming an extrapolation number of 1·0.

B. *Lung metastases of tumours in dogs*

The lung metastasis model can also be applied for determination of growth rate of animal tumours. It was already known that the growth of the lung metastases in the dog and the cat could be very rapid, with rates intermediate between those of human tumours and of transplantable tumours in small laboratory animals. With help of a group of interested veterinary surgeons it was possible to examine

a series of dogs and cats suspected of metastases in the lung.

Many different tumour types were found, including mammary carcinoma, bronchial carcinoma, osteosarcoma and various forms of leukemia. With an interval of about 1 week successive radiographs were made and from these the doubling time of the lung metastases could be determined in 10 animals.

In 2 animals it was possible to give single doses of radiation to determine volume reduction curves.

C. *Lung metastases of an adenocarcinoma transplantable in mice*

We considered it of interest to determine also the growth of lung metastases in laboratory animals. In view of the absence of factors which might cause the decelaration of the growth frequently observed in subcutaneous tumours, it seemed possible that the study might be carried out entirely during exponential growth of the tumour.

We used a poorly differentiated adenocarcinoma mammae of the mouse (M 8013). The cells have a cuboid structure and still show an arrangement in cords; locally there are indications of tube formation. The mitotic activity was high. The tumour had originated in 1950 after administration of estrone to a male C57Bl mouse and because of their better breeding properties it was maintained in the F_1 hybrid of the C57 Bl and CBA.

Only hybrid females were used at an age of 3 months. It should be noted in this connection that spontaneous mammary tumours do not occur either in the C57Bl or in the CBA strains. After 20 subcutaneous passages, no histological alteration in the tumour had occurred. From this subcutaneously growing tumour a suspension of tumour cells was made containing 10,000 single tumour cells per 0·2 ml Ringer's solution. This volume was injected into the tail vein of female mice. Ninety out of the 100 mice developed artificial lung metastases. The intravenously inoculated mice were subjected to radiography of the thorax every day. From the 15th day after intravenous injection, metastases could be seen on the radiographs. The number of metastases per animal that could be demonstrated radiographically varied from 1 to 6. The mice with artificial metastases died spontaneously between the 23rd and the 28th day after the injection of tumour cells. At death the lungs of these mice had been replaced almost completely by tumour tissue.

All the mice were from highly inbred strains and although immunological differences between tumour and host would not seriously upset the work, because all experiments were carried out with similar conditions, it was nevertheless attempted to determine whether such differences could be detected.

After application of heavily irradiated cells all the mice developed a subcutaneous tumour after injection of non-irradiated tumour cells, in the same time interval as the mice without pre-treatment. In this way no evidence for tumour specific antigens could be detected.

In 47 of the 90 animals one or more lung metastases were circular with sharp margins, rendering them measurable through enlarged projection on a screen. The single-dose radiation was administered through two opposing fields; the treatment was a whole body irradiation. Twelve out of the 47 mice with measurable metastases were irradiated on the 15th day or on the 17th day, with a dose of 210 rads (250 kV, HVL 2 mm Cu). The dose was calculated in the midline in the same way as in man and the dog. After measuring the dose by means of LiF dosimetry, a good agreement was found between the measured dose and the calculated dose. After the single dose of radiation the mice were examined radiographically every day and the diameters of the metastases were measured.

With tritiated thymidine incorporation and autoradiography the cell kinetics of the subcutaneous tumour and the lung metastases of the mouse tumour were studied both before and after irradiation. There was a good agreement (Table 2) of the labelling indices in subcutaneous tumours and lung metastases both *in vivo* and using the *in-vitro* technique Steel described [5].

Table 2.

	LI *in vivo*	LI *in vitro*
Subcutaneous tumour	43·9	43·3
Lung metastasis	44·1	44·0

RESULTS

A. *Effects of irradiation on metastases in patients*

After the irradiation a distinct decrease in the volume of metastases occurred in all cases and could be followed from day to day. Thereafter the tumour resumed its growth and the rate of increase of the volume has been measured.

In all cases except one, the decrease in volume of the metastases was followed by a period of regrowth at a higher rate than before irradiation (Fig. 1). After this accelerated increase of the volume, the growth rate gradually returned to the pre-irradiation growth rate. It was not entirely clear what significance we could attach to this period of accelerated growth. Our first experiment with single-dose irradiation was carried out at the time when there were not yet any clear reports in the literature concerning changes of the growth rate of tumours under the influence of irradiation. We found that the greater the reduction in volume the more distinct was the initial acceleration of growth (Fig. 2).

It is of interest to note that the phenomenon of accelerated growth also occurs after a series of fractionated irradiation with a daily dose of 200 rads. That this phenomenon has not been noted before is probably due to an insufficient number of radiographs being made shortly after irradiation.

In analyzing a curve such as that of Fig. 1, it is clear that the fractional decrease in volume found by extrapolation of the straight line after the irradiation may be found in two ways. Backward extrapolation gives the "effective volume decrease" if we assume that growth is resumed immediately after the radiation-induced mitotic delay has elapsed. It is possible however that the lowest point of the curve provides a much better measure of the actual volume decrease, because it represents the remaining tumour volume after the elimination of almost all the killed material.

However, because of the period of accelerated growth its benefit to the patient is entirely lost. It seems advisable, therefore, in determining volume reduction curves of tumours in human patients to use the "effective volume reduction" until the significance of the period of accelerated growth has been fully elucidated. The lowest point in the growth curve following treatment is determined not only by the number of tumour cells that are killed by the radiation and the growth rate of the remaining "viable" tumour cells, but also by the rate at which the killed tumour cells are eliminated. As can be seen in Table 3 the lowest point of the curve is reached 3 to 7 days after the radiation, which indicates that the killed cellular material was eliminated rapidly.

This is not surprising in view of the good vascularization of metastases in the lung. One patient with lung metastases of an embryonal carcinoma of the testicle, with a volume doubling time of 21 days, was treated with a single-dose irradiation of 700 rads; 15 days after irradiation the metastases had disappeared completely. Four years after treatment there is still no sign of recurrence. If but a few tumour cells had been vital after irradiation there should have been a recurrence within 2 years considering the high growth rate of the tumour. We cannot but assume that the single-dose irradiation has cured this patient.

Table 3.

Volume doubling time (days)		% of tumour volume remaining after irradiation				$D_{0\ \text{vol.red.}}$ (rads)	$D_{0\ \text{vol.red.}}$ calc. by $(220+10\ T_d)$	Lowest point of the curve (days after irradiation)	Accelerated growth
		300 rads	500 rads	600 rads	700 rads				
(1)	17		22			330	390	5	+
(2)	21				0		440		
(3)	30			15		310	520	6	+
(4)	39	57				530	610	7	+
(5)	57			38		610	790	7	+
(6)	66		44			600	880	3	+
(7)	82	74			30	440	1040	4	+
(8)	184			73		1680	2060	5	+
(9)	185			76		2150	2070	6	+
(10)	191			72		1810	2130	7	+
(11)	228			78		2320	2500	7	+

The results of Table 3 show that the volume reducing effect increases with increasing dosage. It was found further that with increasing doubling time of the metastases the radiosensitivity expressed in terms of $D_{0\ \text{vol. red.}}$ (volume reduction) decreased. In one patient (Fig. 2) two points of the curve could be determined (Fig. 3). The higher dose caused the greater volume reduction, and the shape of the volume reduction curve resembles a cell survival curve, consisting of a "shoulder" and an "exponential" part. As the volume reduction of the tumour in the case of the

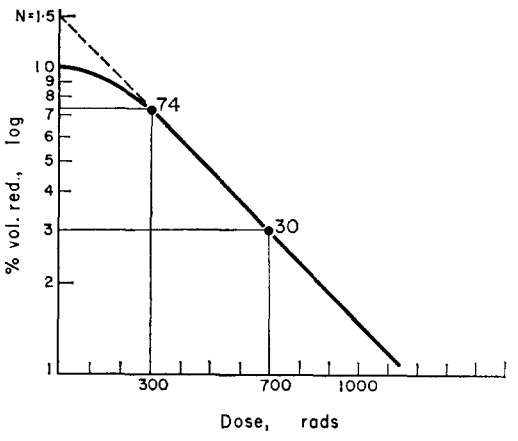

Fig. 3. Tumour reduction curve of the lung metastases of the patient from Fig. 2.

lowest administered dose was only 26%, we may not assume that in this case the shoulder region of the curve has been passed.

In two patients it was possible to treat their pulmonary metastases with three times the same dose of radiation with different time intervals between the doses.

The first patient (Fig. 4) had a bronchial carcinoma with a volume doubling time of 82 days. He was treated with three times 300 rads; the interval between the first and the second dose was 5 days, that between the second and the third dose was 7 days. In the time interval between the first and the second dose no regrowth of the volume was observed. Between the second and the third dose the lowest point of the curve was reached after 5 days.

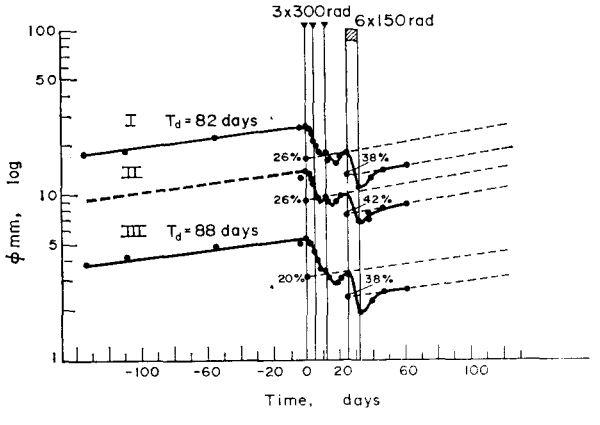

Fig. 4. Influence of different fractionation schemes on the growth of a lung tumour.

The three doses of 300 rads caused an "effective" volume reduction to 20 to 26%, assuming that 13 days after the last irradiation the growth rate of the tumour is the same as before irradiation. From Fig. 4 it can be deduced that the effects of the three doses are not equal. The third dose seems to be less effective than the second one and probably than the first one.

Thirteen days after the last irradiation with 300 rads a series of 6 times 150 rads was given. This course of fractionated irradiation gave an effective reduction of the volume to 38 to 42%. That means that 900 rads given in 7 days overall time gives a smaller volume reduction than 900 rads in 11 days overall time indicating that the size of the fraction is more important than the overall time in which the total dose is given.

The second patient (Fig. 5) had an embryonal carcinoma of the testicle with pulmonary metastases which grew with a volume doubling time of 18 days. Three times a dose of 400 rads was given. The interval between the first and the second dose was 3 days and the interval between the second and the third dose was 7 days. Nine days after the last dose of 400 rads, the lowest point of the curve was reached

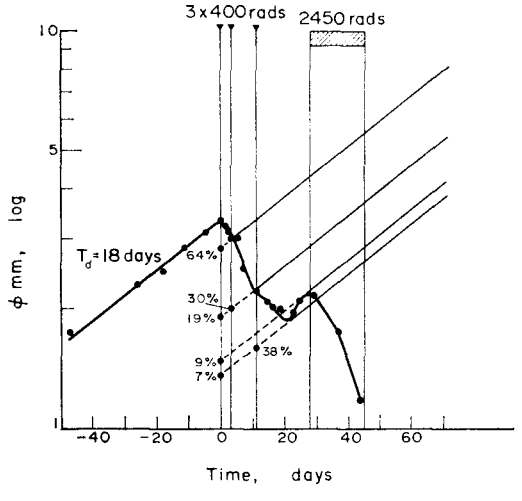

Fig. 5. Influence on the growth of rapidly growing lung metastases of three times 400 rads with different intervals between the fractions. The second fraction seems to be most efficient.

and the three doses together caused an effective volume reduction to 9% of the original volume. The minimum reached after the first dose indicates that the first dose caused a reduction to 64% of the original volume. The first and second dose together caused in the same way a reduction to 19% of the volume. The three doses together show a reduction to 7% of the original volume.

It can be calculated that the second dose of 400 rads caused a reduction to 30% measured from the day that the second dose was given and in the same way the third dose gave a reduction to 38%. It can be concluded that the second and the third doses have a greater effect with regard to volume reduction than

the first dose. This patient was treated in addition with a course of fractionated radiation at a total dose of 2500 rads in 2½ weeks; during this therapy the metastases disappeared and to date have not reappeared again.

B. *Lung metastases of tumours in dogs*

For the series of metastases in dogs, values from 8 to 31 days (Table 4) were calculated. As was to be expected, the doubling times were shorter than in most human metastases, although in men doubling times shorter than 10 days have been recorded as well.

In two animals it was possible to give single doses of radiation and, in accordance with our findings in human lung metastases, the smallest tumour volume was reached shortly after irradiation (Table 5). The elimination of

In one animal (Figs 6 and 7) it was possible for two groups of metastases with the same growth rate to be irradiated with different doses; one group of metastases was irradiated with a dose of 150 rads and the other group with 300 rads. The slight effect of the dose of 150 rads on the volume was to be expected in view of the doubling time of 27 days, a long doubling time in this series. Although a "tumour reduction curve" was made up, we can not be sure that both points were on the exponential part of the curve (Fig. 7).

C. *Lung metastases in mice*

Of the artificially induced pulmonary metastases in mice between the 15th and the 23rd day after injection, the mean volume doubling time was 24 hr.

Table 4.

	Dogs	Sex	Age (years)	Histological diagnosis	Measured volume doubling time (days)
(12)	Shepherd	♀	13	Adenocarcinoma of the adrenal	8
(13)	Shepherd	♀	12	Sarcoma of the nipple	9
(14)	Poodle	♂	12	Malignant synovioma	10
(15)	Setter	♂	12	Carcinoma of the bronchus	10
(16)	Shepherd	♀	11	Carcinoma of the nipple	16
(17)	Bastard	♀	10	Carcinoma complex of the nipple	22
(18)	Terrier	♀	13	Squamous cell carcinoma of the nipple	27
(19)	Pointer	♀	10	Carcinoma of the nipple	28
(20)	Bouvier de Flandres	♀	8	Adenocarcinoma of the bronchus	30
(21)	Spaniel	♀	10	Carcinoma of the bronchus	31

Table 5.

	Volume doubling time (days)	% of tumour volume remaining after irradiation			$D_{0\ vol.red.}$ (rads)	$D_{0\ vol.red.}$ calc. by $(220+10\ T_d)$	Lowest point of the curve (days after irradiation)	Accelerated growth
		150 rads	300 rads	500 rads				
(14)	10			12·5	240	330	6	+
							5	—
(18)	27	76	55		445	470	6	+

destroyed cellular material was largely completed in 6 days. Thereafter the volume of the metastases increased again, with a growth rate that exceeded that observed prior to the irradiation, with the exception of one case in which a dose of only 150 rads was given. In this case, no increase of the growth rate after the irradiation could be demonstrated. After single-session irradiation, the 2 dogs were followed radiographically for at least 3 weeks. Although the observation periods were short it was found also in these animals that, after the initial acceleration, the growth rate gradually returned to its pre-irradiation value in the same manner as described for the human patients.

Within 10 hr after irradiation with 210 rads, tumour volume was found to decrease (Fig. 8). The curve reached its lowest point 45 hr after irradiation, following which the volume started to increase again. Eighty-five hours after irradiation the growth rate measured by the diameter of the lung metastases had returned to its pre-irradiation value. The radiation response can be expressed again as the effective volume reduction of the tumour, obtained by extrapolating the growth curve after irradiation to the day of the radiation.

In this way the tumour volume was found to be effectively decreased to 7% of its original volume. Ten mice with pulmonary metastases

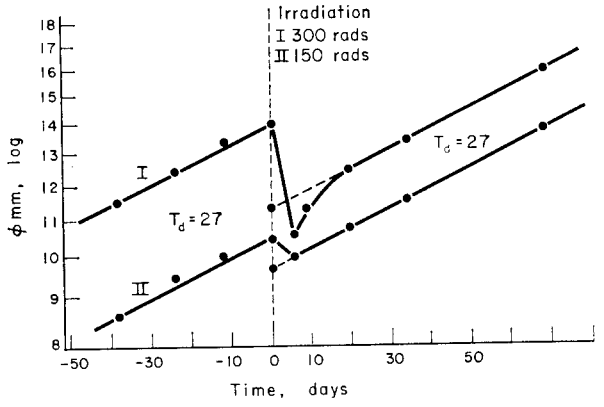

Fig. 6. Influence of two different doses of radiation on the growth of lung metastases in the dog (patient 18, Table 4).

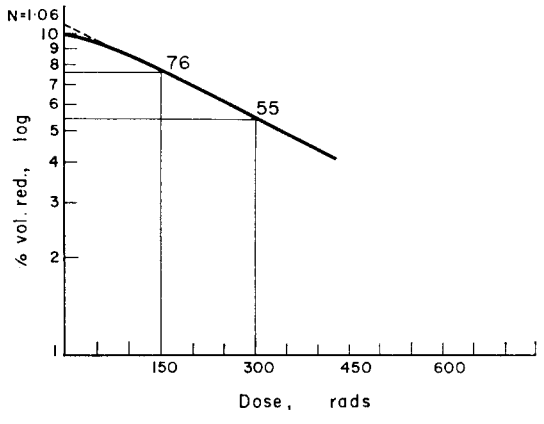

Fig. 7. Volume reduction curve of the lung metastases of the dog from Fig. 6.

were irradiated with 105 rads and that dose gave an effective volume reduction of the tumour to 28%. Drawing a volume reduction curve and assuming that the point representing the lowest dose of irradiation is situated on the exponential part of the curve we find a $D_{0\ \text{vol. red.}}$ of 75 rads and an extrapolation number of $N = 1{\cdot}12$ (Fig. 9). With the smaller dose the lowest point of the curve was reached 24 hr after irradiation and 45 hr after the irradiation the initially determined growth rate was attained again.

As can be seen in Fig. 8, the growth of the metastases after the lowest point of the curve is reached is accelerated considerably. By drawing tangents to the curve obtained, we can estimate between 45 and 53 hr after irradiation, a doubling time of about 8 hr.

Because of the good agreement of the labelling indices in subcutaneous tumours and lung metastases we determined by, the measurements of PLM curves, the inter-mitotic time of the proliferative cells of subcutaneously growing tumours before irradiation with 210 rads and 45 hr after the irradiation, in the period of accelerated growth of the lung metastases.

It can be inferred from the values obtained that the mean cell cycle is shortened in all its components after irradiation, but also that the proliferative fraction has increased to 100% (Table 6). About 80 hr after the single-session irradiation the tumours once more showed the same cell kinetic characteristics as before irradiation (Table 7). At the same moment the growth curve also shows a volume doubling time that is the same as that observed prior to irradiation.

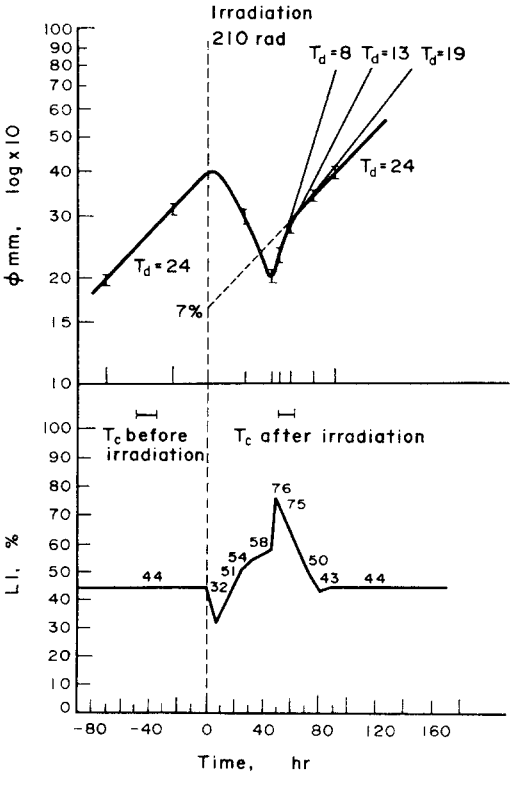

Fig. 8. Influence of a single dose of radiation on the growth of pulmonary metastases in mice. The periods in which the cell cycle times were determined are indicated and a diagram is given of the labelling indices of the subcutaneous tumour before and after the same dose of radiation.

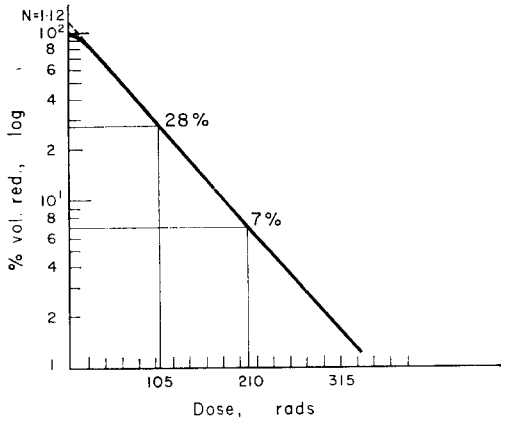

Fig. 9. Volume reduction curve of the lung metastases of the mouse tumour from Fig. 8.

Table 6.

		Unirradiated tumours	Irradiated tumours
Volume doubling time (hr)	T_d	24·0	12·1 (9–19)
Cell cycle time (hr)	T_c	14·5	11·1
Pre-synthesis time (hr)	G_1	2·7	2·1
DNA synthesis time (hr)	T_s	9·2	7·8
Post-synthesis time and mitosis (hr)	G_2+M	2·6	1·3
Labelling index	LI	0·44	0·76
Potential doubling time (hr)	T_p	16·0	10·1
	λ	0·765	0·99
Number of prol. cells per division	α	1·87	2
Growth fraction	f	0·87	1
Cell loss factor	ϕ	0·33	0·19

Table 7.

	LI subc. tumour	LI lung metastasis
Before irradiation	43·9	44·1
7–10 hr after irradiation	32·8	40·6
22–25 hr after irradiation	51·6	80·0
32 hr after irradiation	54·3	61·6
45 hr after irradiation	58·7	56·4
50 hr after irradiation	75·5	50·5
72 hr after irradiation	50·6	—
80 hr after irradiation	43·8	44·3

DISCUSSION

If we assume that the effect of the irradiation on the tumour volume depends in part on the growth rate of the tumour, then it is of interest to divide the tumours listed in Table 1 and Table 4 in three groups:

- a rapidly growing group with a T_d of 15 to 40 days;
- a group with moderately rapid growth with a T_d of 40 to 100 days;
- a slowly growing group with a T_d of more than 100 days.

If we consider the tumours of each of the three groups as one tumour, it is possible to plot the percentage of volume reduction against the dose applied. In Fig. 10 the points are shown for the three groups of tumours in patients, for the murine mammary carcinoma and for the tumour in one of the dogs.

The points originating from 13 histologically different tumours lie on curves, which as regards shape are comparable with volume reduction curves of only 5 tumours. This supports our hypothesis that growth rate is one of the most important parameters in determining radiosensitivity with regard to volume reduction [4, 6, 7]. One can maintain that the decrease of the tumour volume is not directly correlated with the degree in

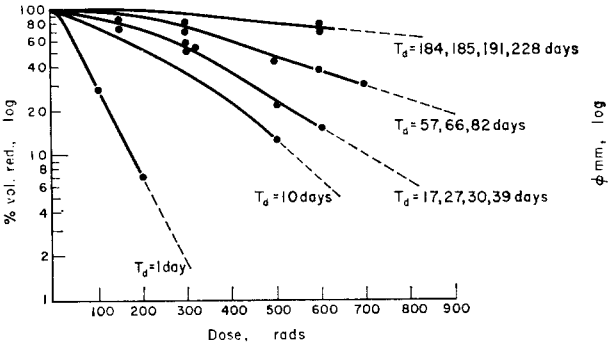

Fig. 10. *Volume reduction curves of various tumours in humans and animals after single-session irradiation with different doses.*

which tumour cells are killed. When the elimination of the killed material after irradiation is hampered, e.g. by oedema, a direct correlation is certainly not expected.

With our pulmonary metastasis model the lowest point of the curve however was always reached within 3 to 7 days, so that the elimination of the killed material must be very rapid and starts directly after the irradiation. Therefore oedema can not play a very important role in these cases. Studies with a rhabdomyosarcoma [8] have demonstrated that although after irradiation a tumour can maintain approximately the same volume, nevertheless only very few tumour cells have retained their

capacity for unlimited proliferation. Therefore one can say that the volume reduction we found after irradiation possibly gives an underestimation of the cellular effects.

It has been postulated by others that volume reduction is not correlated with the chance of local control [9]. In our experiments and also in those of others [7] we found a correlation between dose and volume reduction and also between growth rate and volume reduction per unit dose.

From clinical experience radiotherapists know that in rapidly growing tumour masses like those in Hodgkin's disease, lymphosarcoma or Ewing's sarcoma, it is possible to reach local control with moderate doses of e.g. 4000 rads in 4 weeks. Also in such cases it is known that during radiotherapy the tumour mass decreases rapidly.

In cases of slowly growing tumours, such as highly differentiated squamous cell carcinomas or adenocarcinomas, a much larger tumour dose of e.g. 6000 to 7000 rads, that is just tolerable for the surrounding normal tissues, can not prevent a high percentage of local recurrence. Most of these tumours do not show an appreciable shrinkage during radiotherapy. Therefore the clinicians already had the impression that there must be a crude correlation between growth rate and volume reduction per unit dose caused by radiotherapy and the chance to get a local control.

In nearly all cases we found a period of accelerated growth 3 to 6 days after the irradiation. In the mouse tumour in this period the proliferative fraction was greater than before irradiation and the inter-mitotic time of the proliferative cells had shortened. Synchronization caused by irradiation can in autoradiographical studies give the erroneous result that the proliferative fraction is greater than before irradiation. However this is unlikely because in our investigations also the volume of the tumour increased constantly. Also the knowledge that some of the irradiated cells can divide a few times before death does not give a good explanation of the accelerated growth.

Therefore it seems more likely that after irradiation there is "repopulation" of the tumour at a higher rate, possibly caused by better oxygenation and more space per tumour cell to divide. It is conceivable that also in the human tumours in the period of accelerated growth after a single dose of radiation the proliferative fraction of the tumour has increased causing the higher growth rate. If the hypothesis is valid that radiosensitivity is correlated with growth rate, one could expect a greater radiosensitivity with regard to volume reduction in the period of accelerated growth after irradiation. In order to study if during the period of accelerated growth a given dose can cause a greater reduction in volume as compared with an unirradiated tumour, we treated two patients with three times the same dose of radiation with different time intervals between the soes. In the case of both patients we found that:

1. size of the fraction is more important than overall time with regard to volume reduction.
2. in the period of accelerated growth a dose of radiation can cause a greater volume reduction than the same dose in an unirradiated tumour.

This phenomenon can be of great importance in choosing the most optimal time to apply the second and the following dose fractions. For the selection of that optimal moment for administration of subsequent doses to a tumour, it is of importance to know how the normal tissues will react to such a fractionation scheme.

It is likely that, for most of the tumours treated by radiotherapy, the scheme with daily fractions used in most radiotherapy centers is close to the most effective one. It is however possible that for very rapidly growing or very slowly growing tumours other and less regular fractionation schemes can give better results.

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Experimental Studies on the Mechanisms of Fibrinogen Uptake in a Rat Tumour

H.-I. PETERSON, K. L. APPELGREN and B. H. O. ROSENGREN

Department of Surgery I and Department of Radiotherapy, University of Göteborg, Sahlgrenska Sjukhuset, Göteborg, Sweden

Abstract—*The uptake of intravenously administered labelled rat fibrinogen in a transplanted rat tumour and in different normal tissues of the rat was studied and related to the thromboplastic activity of these tissues. The uptake of rat fibrinogen in tumour was compared to the uptake of intravenously administered labelled human albumin and ^{51}Chromium-EDTA. The results suggest that the uptake of fibrinogen in a tumour is not an active deposition as fibrin due to high thromboplastic activity of tumour tissue, but a passive leakage into the extravascular space due to a high permeability of the tumour vascular wall.*

INTRODUCTION

A THROMBOPLASTIC factor was extracted from human carcinomas by O'Meara [1]. This factor was related to simultaneously observed extravascular fibrin deposition in tumour tissue [2–4]. In experimental studies intravenously administered ^{125}I- and ^{131}I-labelled fibrinogen was taken up in tumours [5–11]. A correlation was found between the thromboplastic properties of different rat tumours and the uptake of labelled heterologous fibrinogen [12].

A comparatively high thromboplastic activity was found in several mouse and rat tumours [13, 14]. However, in these studies a high thromboplastic activity was also found in brain and lung tissue.

O'Meara suggested that fibrin might be a suitable stroma for tumour cell invasion. However, recent experimental studies on fibrinolysis in growth and spread of tumours suggested that fibrin deposition might instead be part of a reaction against the growing tumour [13].

Thus, fibrin deposition or fibrinogen uptake might, in some way, influence the growth and spread of malignant tumours. The uptake of fibrinogen might be influenced by the thromboplastic properties of the tumour, by inhibition of this activity or by fibrinolysis. In addition, the property of tumours to take up intravenously administered labelled fibrinogen might be utilized practically for diagnostic procedures [15].

However, the mechanisms behind the uptake of fibrinogen–fibrin in tumours still remain obscure. Fibrinogen might leak passively from plasma into the extravascular space through tumour capillaries of high permeability or might be concentrated by fibrin deposition in a thromboplastic tissue.

Thus, the aim of the present study was to investigate, in the rat, the uptake of labelled homologous fibrinogen in a transplantable tumour and in different normal tissues and relate this uptake to the thromboplastic properties of the tissues. Furthermore, the uptake of labelled fibrinogen in tumours was compared to the passive uptake in tissues of radioiodinated human serum albumin (^{125}I-RISA and ^{131}I-RISA) and of ^{51}Chromium-ethylenediamine-tetra-acetate (^{51}Cr-EDTA), a small extracellular anion.

MATERIAL AND METHODS

Animals and tumour

Inbred rats from a hooded strain, 3 to 4 months old and with a mean weight of about 200 g were studied. A 20-methylcholanthrene induced sarcoma, propagated in this strain of

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animals, was studied in its 27th and 29th transfer generation. All animals were transplanted with a solid piece of tumour tissue intramuscularly into one hindleg and the experiments were performed about 3 weeks after transplantation with a mean tumour diameter of about 1 cm.

Thromboplastic activity

The thromboplastic activity of tumour tissue, brain, lung, liver and striated muscle was examined on tissue homogenate in dilution with Owren's buffer by measuring the clotting time of rat plasma in a recalcification system with 0·03 M calcium chloride. This method was described in detail previously [13]. Control clotting time without tissue homogenate was 110 ± 5 sec.

Isotopes

Each animal was injected intravenously in a tail vein with 1 ml of isotope solution containing one or two of the radioactive tracers in saline and in amounts given below.

a. *Fibrinogen.* Homologous rat fibrinogen was used. The preparation and labelling of this fibrinogen was described earlier [11]. A batch of 6 ml of a 1% rat fibrinogen solution was labelled with 2 mCi ^{125}I and diluted to a final volume of 32 ml. Each animal was given 1 ml of this solution, corresponding to 2 mg of fibrinogen with 10 μCi ^{125}I. The clottable activity of the labelled fibrinogen was 85% and the $t_{\frac{1}{2}}$ 26 hr.

b. *Labelled albumin.** For practical reasons *human* albumin labelled with ^{125}I or ^{131}I (^{125}I-RISA or ^{131}I-RISA) was used. Each animal was given 3 μCi of ^{131}I-RISA or 5 μCi of ^{125}I-RISA.

c. *^{51}Chromium - ethylene - diamine - tetra - acetate (^{51}Cr-EDTA).** ^{51}Cr-EDTA is a monovalent extracellular anion with capillary exchange properties practically identical to those of sucrose [16]. Each animal was given 5 μCi of ^{51}Cr-EDTA.

Radioactivity measurements

Measurements of one or two isotopes in tissue and blood samples were made in a well scintillation counter with a two channel pulse analyzer. At least 10^4 counts were registered on each channel. Due correction was made for background and channel interference. The activities in cpm/ml blood and cpm/g tissue were calculated. Hematocrit was used to calculate the activity per ml plasma assuming zero activity in erythrocytes. Since all animals were given the same dose of isotope and since animal and tumour weights were slightly varying, all data are presented as ratios between tissue and plasma activity.

*Obtained from AB Atomenergi, Studsvik, Sweden.

EXPERIMENTS

1. *Thromboplastic activity of tumour and normal tissues.*

Procedure. Twelve determinations on six different specimens of tumour, brain, lung, liver and striated muscle were made. The thromboplastic activity of tissue homogenate in a dilution of 1:10 with Owren's buffer was examined.

Results. The results are presented in Table 1. A high thromboplastic activity (short clotting time) was found in brain and lung tissue. The thromboplastic activity of tumour tissue was comparatively low but still significantly higher than the activity in muscle, into which tissue tumours were transplanted. A low activity was found in liver.

Table 1. Thromboplastic activity of tumour (20-methylcholanthrene induced rat sarcoma) and various normal tissues of the rat. Mean values of 12 determinations from each tissue. A low clotting time means a high thromboplastic activity.

	Clotting time (sec.)	
	Mean	S.D.
Brain	28	2
Lung	20	1
Liver	64	8
Muscle	54	3
Tumour	41	5

2. *Uptake of labelled rat fibrinogen in tumour and normal tissues.*

Procedure. Thirty rats transplanted with the 27th transfer generation of the 20-methylcholanthrene induced sarcoma were studied. All animals were injected intravenously with 1 ml of the labelled fibrinogen solution. Groups of 10 animals were sacrificed by exsanguination under ether anaesthesia after 2, 8 and 20 hr respectively. One millilitre of blood was drawn from each animal and biopsies from macroscopically vital tumour, brain, lung, liver and muscle were taken. The activity in blood and tissues was registered and calculated as cpm/ml plasma and cpm/g tissue.

Results. Ratios between the activity/g of tissue and the activity/ml of plasma are given in Fig. 1. Mean values $\pm$ 1 S.D. (n = 10) are presented.

These ratios of different normal tissues to plasma increased rapidly to a certain value after 2 hr (brain: 0·011, lung; 0·23, liver: 0·11, muscle: 0·012) and did not change significantly during the following observation time. On the contrary the tumour–plasma ratio increased continuously during the experiment.

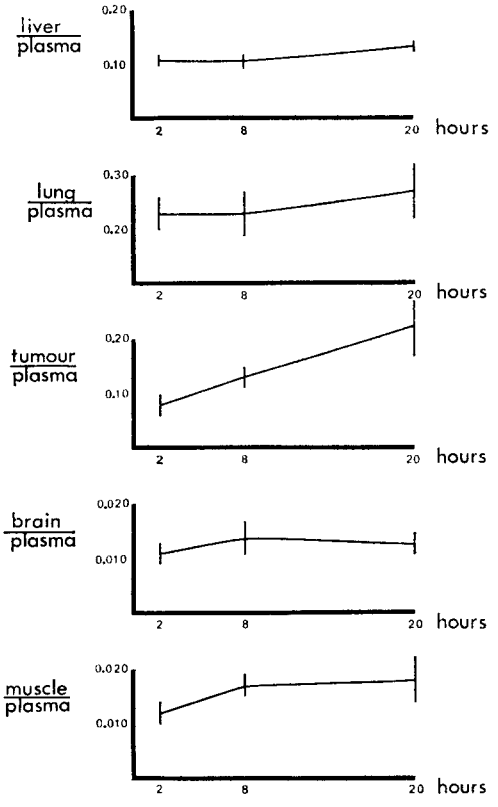

Fig. 1. *Tissue–plasma ratios for labelled fibrinogen in tumour and various normal tissues of the rat. Note the continuous increase in tumour, while ratios in normal tissues are practically unchanged during the experiment. Mean values of* 10 *determinations with S.D. at each time are given.*

3. *Uptake of labelled human albumin and of* ^{51}*Cr-EDTA in tumour and muscle.*

Procedure. Fifty animals transplanted with the 29th transfer generation of the 20-methylcholanthrene induced sarcoma were studied. Twenty-five animals were injected intravenously with 1 ml solution containing 3 μCi of ^{131}I-RISA and 25 animals were injected with 1 ml solution containing 5 μCi ^{125}I-RISA and 5 μCi ^{51}Cr-EDTA. Five animals from each group were sacrificed 1, 2, 4, 8 and 20 hr respectively after the injection. From each animal 1 ml of blood was drawn and biopsies of macroscopically vital tumour tissue and muscle were taken. The activity as cpm per ml plasma and per g tissue was calculated.

Results. Ratios between the activity of the different isotopes in tumour and muscle compared to plasma are presented in Fig. 2 as mean values ± 1 S.D. (n = 5). Tissue–plasma ratios for ^{131}I-RISA and for ^{125}I-RISA were practically identical both in tumour and muscle. The tumour/plasma ratio for RISA increased to 0·12 after 1 hr and then increased continuously as did the ratio for fibrinogen in experiment 2. At the 20 hr point the ratios for RISA and for fibrinogen were within the same range. The muscle–plasma ratio for RISA increased to 0·010 after 1 hr and then increased continuously, while the ratio for fibrinogen in experiment 2 was practically constant throughout the experiment. The ^{51}Cr-EDTA activity in plasma and tissue decreased very rapidly. Therefore, the 1-hr ratio was used and suggested to give a fairly good estimation of the equilibrium ratio between tissue and plasma

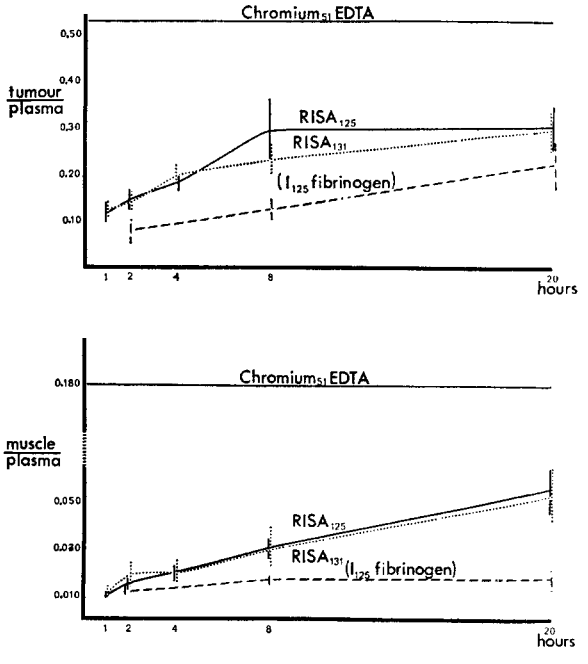

Fig. 2. *Tissue–plasma ratios for* ^{125}*I-RISA and for* ^{131}*I-RISA in tumour and muscle. Mean values of* 5 *determinations with S.D. at each time. The* 1 *hr tissue–plasma ratio for* ^{51}*Cr-EDTA is included as a horizontal straight line. Values on fibrinogen from experiment* 2 *are given for comparison. Note that the continuous increase of RISA ratios in tumour is practically identical for* ^{125}*RISA and for* ^{131}*RISA and parallel to the increase of fibrinogen ratios. In muscle a continuous increase at low ratio values is observed for RISA but not for fibrinogen.*

for this isotope. This ratio is indicated in Fig. 2 as a horizontal straight line giving an estimation of the extracellular space of the tissue which thus amounted to 0·18 ml/g in muscle and to 0·53 ml/g in tumour tissue.

DISCUSSION

The thromboplastic activity of a rat tumour was found to be significantly higher than the activity of normal striated muscle, that is, the tissue into which this tumour was transplanted and growing. However, an even higher thromboplastic activity was found in brain and lung tissue of the rat. After intravenous administration of labelled homologous fibrinogen, the tissue–plasma ratios indicated a continuously increased activity in tumour tissue between 2 and 20 hr. This would suggest a progressive localization of fibrinogen to the tumour. A similar increase of fibrinogen was not found in brain or lung tissue in spite of the high thromboplastic activity of these organs. This might indicate that the localization of intravenously administered fibrinogen into a normal tissue is independent of the thromboplastic activity of that tissue. The normal tissues reached their maximum ratio within 2 hr and the ratio then remained fairly constant. The fast equilibration in normal tissues indicated intravascular localization, e.g. with a tissue–plasma ratio of about 0·015 ml/g in skeletal muscle, which gives an estimation of the plasma space of muscle [17, 18]. The plasma space of tumour tissue would then be about 0·08 ml/g according to the 2 hr ratio for fibrinogen.

The progressive uptake of fibrinogen in the thromboplastic tumour might depend upon other mechanisms than fibrin deposition such as the passive leakage of fibrinogen from plasma into the extravascular space. With this mechanism the failure to show progressive uptake into the thromboplastic brain and lung tissue might be explained by the incapacity of the large fibrinogen molecule to pass the blood–tissue barrier of these tissues.

Therefore, the uptake of intravenously administered RISA and ^{51}Cr-EDTA in tumour and muscle was studied and related to the uptake of homologous fibrinogen. After the fast increase during the first hour a continuous increase of the RISA tissue–plasma ratio was found in tumours. This increase was nearly parallel to that of fibrinogen. At the end of the experiment the difference between ratios of fibrinogen and of albumin was not significant ($p > 0{\cdot}05$). The parallelism of ratios for RISA and for fibrinogen, where the ratio of albumin was always higher than that of fibrinogen, indicates that no concentration of fibrinogen over that of albumin took place. Albumin uptake is evidently not influenced by the thromboplastic activity of tumour tissue but is a passive leakage of protein from plasma into the extravascular interstitial space.

The tissue–plasma ratio for albumin in muscle was low but increased continuously. This increase was not seen for fibrinogen. The difference is probably explained by the lower permeability of the capillary wall of muscle for the large fibrinogen molecule. However, even the permeability of albumin through the muscle capillary wall is known to be low [19]. ^{51}Cr-EDTA does not penetrate the cell membrane but equilibrates rapidly with the extracellular space. Thus, the tissue–plasma ratio of this isotope will give an estimation of the extracellular space of tumours and normal tissues. An extravascular space of 0·18 ml/g in skeletal muscle is in agreement with published values for other small, water-soluble extracellular molecules and ions [20]. The ratio of ^{51}Cr-EDTA for tumour tissue indicates a much larger extravascular space (0·53 ml/g) including plasma space about 0·10 ml/g. The ^{51}Cr-EDTA ratio would indicate the maximal possible value for fibrinogen or albumin with passive leakage of protein to equilibrium between extracellular space and plasma. Thus, the concentration of radioactive fibrinogen and albumin in the extravascular interstitial space of the tumour did not reach the concentration of plasma within the observation time.

The results of the present experiments might indicate that the localization of fibrinogen to tumours is not an active deposition due to the high thromboplastic activity of tumour tissue, but might more likely be an inactive leakage to the extravascular space based on a high permeability of the tumour capillary wall.

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Consistent Chromosome Abnormalities in Each of Three Cases of Childhood Lymphosarcoma*

BAYZAR ERKMAN-BALIS† and P. E. CONEN

The Department of Pathology and the Research Institute, The Hospital for Sick Children, Toronto, and The University of Toronto, Canada

Abstract—*Chromosome analyses on multiple tissues from three children with lymphosarcoma revealed a consistent chromosome abnormality for each tumour. In Case 1, direct examination of cells from a supraclavicular lymph node revealed a normal cell type and two different main cell types with 47 chromosomes and related cell types with 48 and 49 chromosomes. A normal chromosome complement was found in peripheral blood and bone marrow aspirate. An axillary lymph node, removed at autopsy five weeks later, was cultured for 11 hr and contained the same five cell types but in different proportions. In Case 2, cells with 46 to 49 chromosomes were found in a tumour involving the kidney. In Case 3, cells with pseudodiploid karyotype were found in a tumour involving ovaries and bone marrow on direct examination and after 24 hr in culture. The karyotype of peripheral blood was normal. The findings in Cases 1 and 3 provided evidence of preservation of the same chromosome abnormalities in different tumour sites within the individual. The chromosome abnormality was present in short term cultures in Cases 1 and 3, suggesting that brief periods of culture can be used to increase the number and quality of mitoses. The karyotypes of all three cases were near-diploid or pseudodiploid.*

INTRODUCTION

CHROMOSOME abnormalities have been found in several human leukaemias and tumours [1, 2]. The abnormal karyotypes in lymphosarcoma have been frequently pseudodiploid or near diploid [3–15].

This report describes abnormal chromosome patterns in three lymphosarcomas. The first tumour included cells with diploid complement and cells containing 47 chromosomes with two distinct karyotypes; the second tumour had cells with diploid complement and cells with 49 chromosomes and the third tumour had a pseudodiploid chromosome pattern.

*This study was supported by The Ontario Cancer Treatment and Research Foundation.

†Present address: Department of Pathology, Loyola University, Stritch School of Medicine, Maywood, Illinois, U.S.A.

MATERIAL AND METHODS

Specimens consisted of surgically excised tumours and lymph nodes, marrow aspirates, peripheral blood and samples of spleen removed shortly after death. Mitotic cells from tumours, lymph nodes and bone marrow were examined by a direct method without prior *in vitro* growth. Metaphases were also studied from short term [8–48 hr] and long term (several weeks) cultures of cells from tumour or lymph nodes in media 199 containing 20% foetal calf serum, penicillin and streptomycin. Blood samples were cultured by standard methods and spleen cells were processed by a modification [12] of the blood culture technique.

All cells were fixed in 1:3 glacial acetic acid/methanol, air dried and stained with carbol fuchsin. Chromosomes were counted and analysed (into groups) from photomicrographs

aided by direct observation of the cell under a microscope. Karyotypes of many cells were prepared from each specimen (Table 1).

CASE REPORTS

Case 1

This 7-year-old boy had lethargy and a two-week history of intermittent abdominal pain and distension. Examination revealed generalized lymphadenopathy, a right-sided pleural effusion, ascites, distended abdominal and leg veins, hepatomegaly and an abdominal mass in the right lower quadrant.

Investigations of blood and marrow were normal. A supraclavicular lymph node was biopsied and a diagnosis of lymphoblastic lymphosarcoma was made (Fig. 1).

The patient was treated with prednisone, procytox and radiotherapy. He died 6 weeks after admission. Autopsy revealed involvement of skin, lymph nodes, liver, oesophagus, stomach, serous cavities by lymphosarcoma and there were groups of undifferentiated cells, presumed to be lymphosarcomatous, in sections of the bone marrow from the upper end of the femur.

Case 2

This 12-year-old boy was investigated for intermittent pain in the left flank, haematuria, burning and urgency for four months and was found to have a tender mass in the left upper quadrant of the abdomen. Investigations of blood and marrow were normal. There was microscopic haematuria, and intravenous and retrograde pyelograms revealed left-sided hydronephrosis and a space-occupying lesion in the left renal pelvis.

At laparotomy (May 1964) the left kidney and tumour (combined weight 150 g) and the left para-aortic lymph nodes were removed. Microscopic eaxmination revealed lymphosarcoma (Fig. 2). Examination of the para-aortic lymph nodes showed only reactive hyperplasia.

The patient was given a course of radiotherapy to the left renal area but in September 1964, a 4×3 cm subcutaneous mass appeared in the surgical scar and was excised. Histological examination disclosed neoplastic tissue (Fig. 3) similar to the original tumour, infiltrating the underlying muscle. Radiographs of the chest and long bones were normal. Further radiotherapy was given and at last examination (January 1968) there were no signs of recurrence and haematologic studies have remained normal.

Case 3

This $5\frac{1}{2}$-year-old girl was investigated for fatigue, swelling of the left upper eyelid, headaches, periumbilical pain and an abdominal mass which arose from the pelvis.

Investigations of blood counts were normal but marrow films contained many immature cells, lying singly or in clumps (Fig. 4) interpreted as metastases. Chest radiographs and a skeletal survey were normal.

Laparotomy in July 1964 revealed a large mass in the region of each ovary (right—$12 \times 6 \times 5$ cm; left—$6 \times 4 \times 4$ cm); these were excised and found to be lymphosarcoma (Fig. 5).

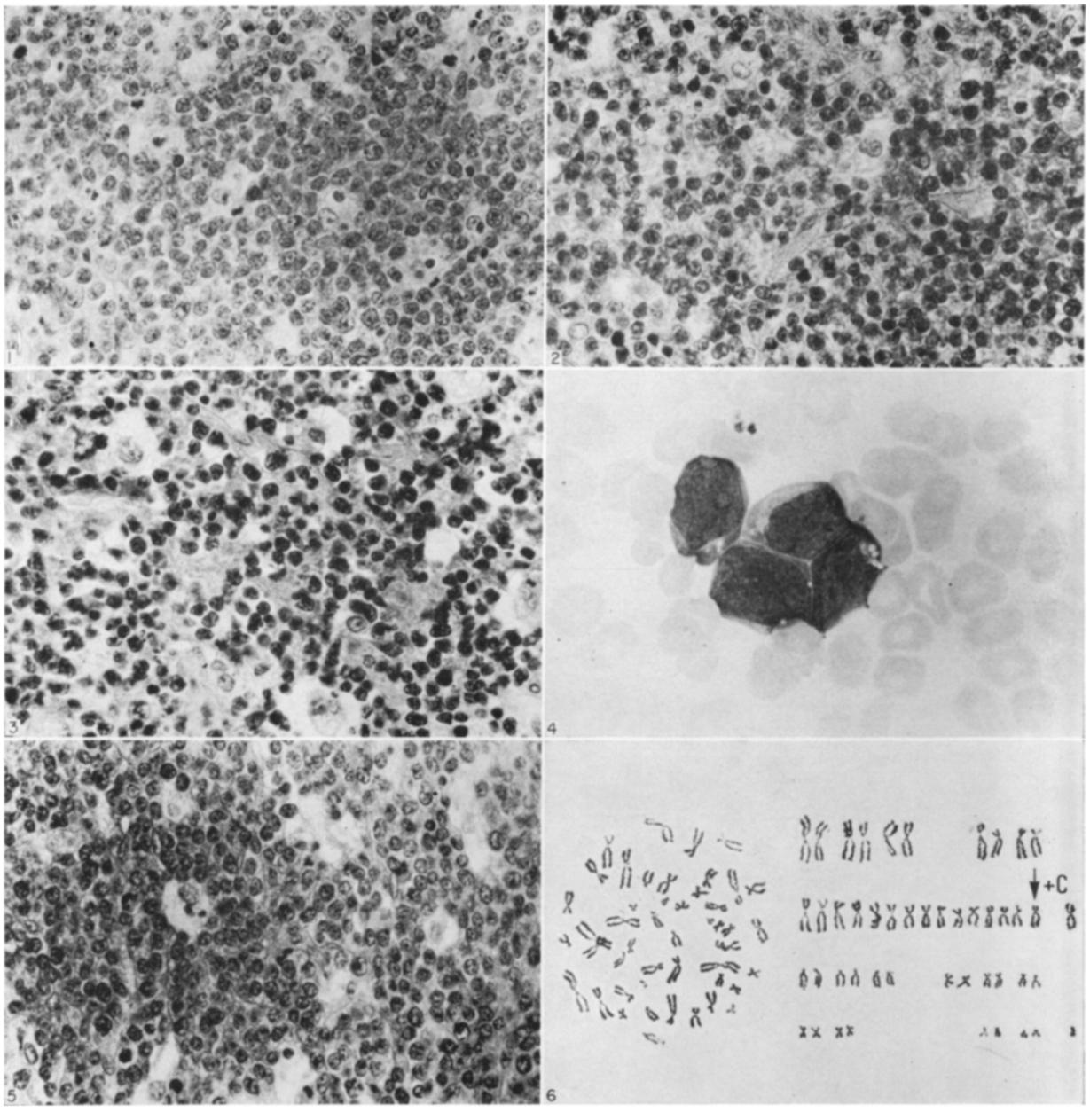
The patient was treated with prednisone, 6-mercaptopurine, systemic and intrathecal methotrexate and radiotherapy. Malignant cells were often identified in marrow aspirates, and in terminal ascites. The patient died in January, 1965. Consent for autopsy was not obtained.

RESULTS

Case 1

The supraclavicular lymph node, excised prior to treatment (T_1), was examined directly without culture and several chromosomally distinct cell types were found (Table 1). Cells with 47 chromosomes including an additional C group chromosome (Fig. 6) were predominant. There were also cells with normal male karyotype, and cells with 47 chromosomes including a small acrocentric marker resembling a G chromosome (Fig. 7). Four cells with counts of 46 chromosomes were presumed to be broken since the additional C or "G" chromosome was present but a different chromosome was lacking from each cell. Four cells with 48 chromosomes also had a consistent pattern which included both additional chromosomes (Fig. 8). One cell with 49 chromosomes contained two additional C chromosomes and the small acrocentric. Analysis of metaphase plates from cultured peripheral blood showed a normal male karyotype.

An axillary lymph node and samples of marrow and spleen were obtained 6 weeks later (T_2), approximately 2 hr after death. Marrow cells examined directly revealed a normal male karyotype. Cells from the lymph node were cultured for 11 hr and chromosome patterns were identical to those found in the original specimen (Fig. 8) but there was a slight change in the percentage of cell types. Cells from spleen cultured with phytohaemag-



Figs. 1 *to* 5 *are photomicrographs of lymphosarcoma from the three cases.*

Fig. 1. *Case* 1. *Supraclavicular lymph node.* (*H & E* × 255).

Fig. 2. *Case* 2. *The mass in the kidney.* (*H & E* × 255).

Fig. 3. *Case* 2. *Subcutaneous mass in the surgical incision.* (*H & E* × 255).

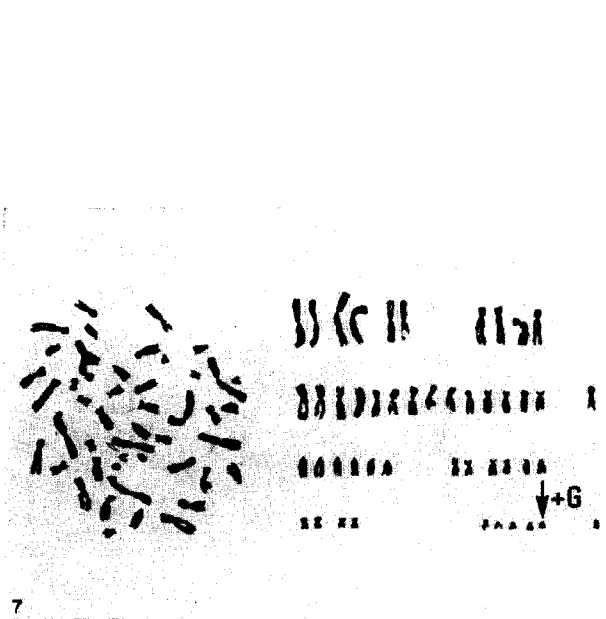
Fig. 4. *Case* 3. *Immature cells presumed to be lymphosarcoma in marrow aspirate.* (*Wright-Giemsa* × 1050).

Fig. 5. *Case* 3. *Ovarian tumour.* (*H & E* × 255).

Figs. 6 *to* 13. *All single karyotypes were prepared from cells examined without culture. Multiple karyotypes have been arranged to show evidence of clonal evolution of karyotype and to allow easy comparison of chromosomes obtained directly from different tissues or after culture. In single and multiple karyotypes: the vertical arrows and letters indicate the group(s) of chromosomes (but not the individual chromosome) by which the cell differs from that of the celltype in the line above; (—) indicates a 'missing' chromosome compared to the cell-type above it; and ringed-arrows indicate structurally abnormal chromosomes.*

Fig. 6. *Case* 1. *Cell from supraclavicular lymph node contains* 47 *chromosomes. The 'additional' chromosome is in the C group but the particular chromosome in this group cannot be identified.*

(to face p. 684)



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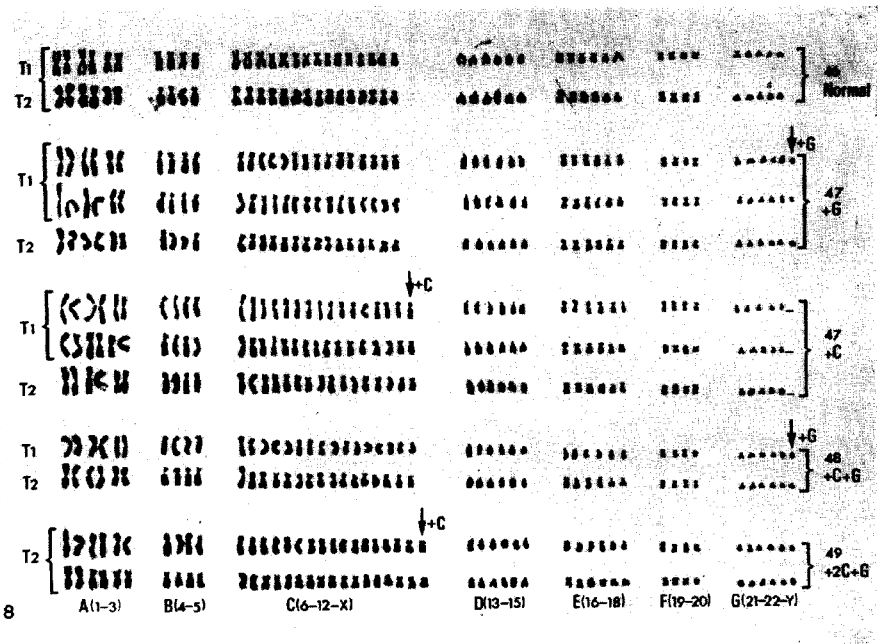

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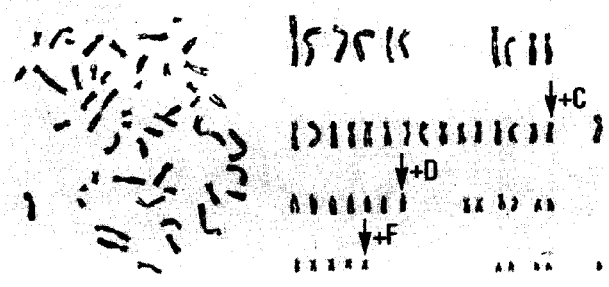

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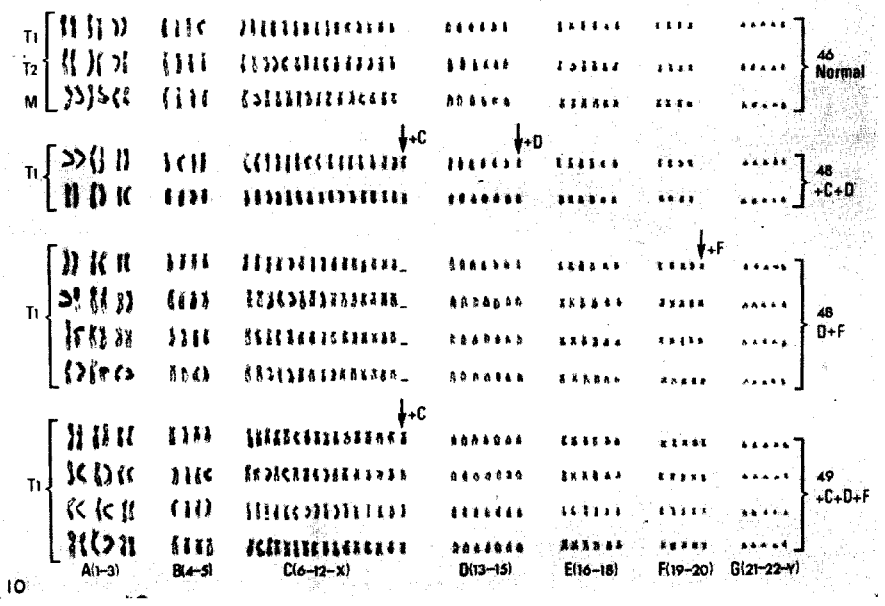

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Fig. 7. *Case* 1. *Cell from supraclavicular lymph node also has* 47 *chromosomes. The 'additional' chromosome is an abnormal marker chromosome and has only been placed in the G group because of its size. It is not the Y chromosome and does not have a characteristic shape.*

Fig. 8. *Case* 1. *Multiple karyotypes from supraclavicular lymph node biopsy* (T_1) *examined without culture and from an axillary lymph node* (T_2) *obtained at autopsy and cultured for* 11 *hr. The five cell types illustrated were identified in both lymph nodes and might represent clonal evolution of karyotype although there are two pathways to* 48 *and* 49 *chromosome cells. Arrows depict groups and not individual chromosomes.*

Fig. 9. *Case* 2. *Cell and karyotype from tumour with* 49 *chromosomes including an 'additional' chromosome in groups C, D and F.*

Fig. 10. *Case* 2. *Multiple karyotype from primary tumour* (T_1) *to show cell types with* 46 *Normal,* 48 (+*C*+*D*), 48 (+*D*+*F*), *and the predominant cell type with* 49 *chromosomes* (+*C*+*D*+*F*). *Cells from direct examination of bone marrow* (*M*) *and direct examination and short term culture of the metastatic lesion* (T_2) *revealed a normal male pattern (exception—see text).*

Fig. 11. *Case* 3. *Cells from tumour revealed a pseudodiploid chromosome pattern. Only one normal #*1 *and five D chromosomes were identified and there was a large submetacentric marker (ring-arrow) paired with #*1 *because of the matching short arm, and a small acrocentric placed in the G group. The first chromosome of the latter group appeared to be smaller in this cell but this was not found in other karyotypes. The long arm of one D chromosome (ring-arrow) was longer than those of the other members of the group (more obvious in Figs.* 12 *and* 13).

Fig. 12. *Case* 3. *Pseudodiploid karyotype from bone marrow is similar to that of the tumour except for prominent satellites on a D chromosome.*

Fig. 13. *Case* 3. *Multiple karyotypes show the consistent pseudodiploid pattern in cells from the tumour and bone marrow examined without culture, and in* 24*-hr cultures* (TC) *of cells from the tumour. Cells from cultured blood (blood TC) were normal.*

Table 1. Tissues examined and chromosome results

Date	Specimen	Histologic diagnosis	Culture (hr)	PHA	Mitotic cells: Total	Ana-lysed	Karyo-types	Chromosome number: 42	43	44	45	46 N	46 Pseudo-diploid	47	48	49	88	92	94
Case 1														G	C				
C.J. Male																			
7 years																			
2.1.64	Lymph node	Lymphosarcoma	Direct	–	109	109	53			1	1	24	4*	29	45	4	1		
31.1.64	Blood	Normal	72	+	41	41	3				3	38							
10.2.64	Marrow†	Normal	Direct	–	30	16	4		1		3	24						1	1
	Lymph node†	Lymphosarcoma	11	–	48	48	37					5		8	29	4	2		
	Spleen†	Normal	55, 72	+	25	16	5			1	3	21							
Case 2																			
J.C. Male																			
13 years																			
1.5.64	Kidney	Lymphosarcoma	Direct	–	75	73	26				2	32			3	10	27		1	
	Marrow	Normal	Direct	–	28	26	4			2	1	24							1	
3.9.64	Skin nodule (surgical scar)	Lymphosarcoma	Direct	–	94	72	16			1	4	86				1			2	
			24 and 48	–	53	41	8			1	5	46				1			1	
			8 weeks	–	26	26	26					5	11		8	1			0	1
Case 3																			
A.M. Female																			
5 years																			
13.7.64	Abdominal mass	Lymphosarcoma	Direct	–	113	80	20	1	1		2		107	2					
			24	–	37	7	7	1	1	2	2		30	1					
			8 weeks	–	41	37	4		1	1	1	34							4
15.7.64	Marrow	Lymphosarcoma	Direct	–	128		36				5		123						
	Blood	Normal	72	+	70	70	9			1	3	66							

*Broken †Specimen obtained 2 or 4 hr (see text) after death

glutinin had a diploid complement except for a few cells in which either chromatid breaks, a long acrocentric marker chromosome, or a dicentric chromosome were seen.

Case 2

Direct examination of cells from the tumour (T_1) revealed two main karyotypes (Table 1): (a) cells with a normal male complement, and (b) cells containing 49 chromosomes with an extra chromosome in groups C, D and F (Fig. 9). In addition, there were intermediate chromosome patterns: ten cells had a chromosome count of 48. In seven of these the additional chromosomes were a D and F, and in two a C and D (Fig. 10). Two cells with a count of 47 had an additional C and F chromosome respectively. These intermediate patterns were interpreted as evolutionary stages of the main abnormal karyotype. Direct examination of the bone marrow revealed only cells with normal male chromosome complement (Fig. 10, line M).

Cells from the metastatic skin lesion (Fig. 10, T_2) examined directly and after 24 and 48 hr of culture revealed a normal male karyotype except for two cells which, like one of the intermediate patterns of the original tumour, had 48 chromosomes with an additional C and D chromosome. Analyses of most cells from 8-week cultures were abnormal. Of 8 cells with 47 chromosomes, 4 cells contained an extra C, and 3 cells an extra F chromosome. There were also four cells with 46 chromosomes containing the extra F but lacking a different chromosome in each cell and a near-tetraploid cell with 94 count including 2 additional chromosomes in the F group. Only 5 cells with 46 chromosomes had normal patterns. The abnormalities in 46-chromosome cells included 5 cells with a marker chromosome in the B group, and 9 cells with an unusually small chromosome placed in the C group. Tetraploidy and endoreduplication were frequent and scoring 414 mitotic cells from 5 slides provided incidences of 22% endoreduplication and 11% teraploidy. Illustrations and a more detailed description of these abnormalities in the 8-week skin cultures are not given because this patient received therapeutic irradiation (2460 rads) four months before biopsy of the skin lesion.

Case 3

Metaphase plates prepared directly from the tumour and marrow revealed a pseudodiploid karyotype (Figs. 11–13). Only a single A_1 chromosome was identified, and five D group chromosomes were present, one of which had an abnormally large long arm. There was a large submetacentric marker chromosome, with a short arm equal to that of an A_1 chromosome, and a small chromosome which resembled a G. Karyotypes of cells from marrow were identical to the tumour except for a prominent satellite on one of the D group chromosomes (Figs. 12 and 13). Cells from 24-hr tumour cultures had an identical chromosome pattern (Fig. 13) but cells from 8-week cultures yielded only mitotic cells with a normal female pattern. Analysis of metaphases from cultured blood showed a normal female karyotype (Fig. 13).

DISCUSSION

Chromosomal abnormalities were present in all 3 cases of lymphosarcoma studied at our hospital (this report), and have been detected in the majority of cases reported in the literature [3–15]. Near-diploid chromosome patterns are frequent in lymphosarcoma [3, 5, 7–12, 15] and Cases 1 and 2 of this report contained 46 to 47, and 46 to 49 chromosomes. In both cases, the cell types in the tumour differed from other cell types by the presence of only 1 chromosome, and might be interpreted as examples of clonal evolution of karyotype, as shown in serial biopsies by Gripenberg *et al.* [14], although the pathway of evolution in our cases was not certain. Pseudodiploid karyotypes are also frequent in malignant lymphomas [3, 4, 7, 11–14] particularly in Burkitt's tumour [5, 10]. Case 3 of the present report had a pseudodiploid karyotype which contained an "extra" G group chromosome, lacked one D and one A group chromosome and had a large submetacentric chromosome. The two latter abnormalities are similar to those found in ascitic cells from a case with a Burkitt-like lymphoma [4]. It should be mentioned that our Case 3 had some histological resemblances to Burkitt's lymphoma.

The chromosomal abnormalities were consistent within tumour cells from different locations in two of our patients. In Case 1 the same abnormal chromosome pattern was found in a biopsied lymph node and in a second lymph node sampled 6 weeks later at autopsy although the percentage of abnormal cell types in the two specimens was different. This difference may be related to the natural progression of the tumour, a selective effect of treatment or other factors including selection of cells for study. In Case 3, the chromosomal abnormality in cells from bone marrow was the

same as that found in the lymph node and correlated with the presence of lymphoma cells in the marrow-aspirate. In Cases 1 and 2, smears and chromosome studies from iliac crest marrow aspirates were normal although cells presumed to be lymphosarcomatous were found in femoral marrow sections taken at autopsy 5 weeks later in Case 1.

Sandberg *et al.* [8] described 6 cases with marrow involvement by lymphosarcoma, and found chromosomal abnormalities in each of the 6 cases. However they did not study the chromosomal pattern in the original tumour. Tjio *et al.* [11] described a pseudodiploid chromosome pattern found on direct examination of cells from lymph node and from marrow of a 35-year-old woman with lymphosarcoma. Lymphosarcoma cells were not identified in smears of marrow aspirate. Jacobs [5] found direct marrow preparations to be chromosomally normal from 4 patients with Burkitt's lymphoma, in whom chromosomal abnormalities were found in tumour samples.

In Case 1, chromosomal abnormalities were found in cells obtained from 11-hr cultures of the lymph node and were the same as those found on direct examination of the lymph node. In Case 3, 24-hr cultures of tumour revealed the same abnormal chromosome pattern as in direct examinations of the tumour but fibroblast-like cells from 8-week cultures yielded only normal chromosomal patterns. Baker [3] also found the same chromosomal abnormality on direct examination and after short term culture of lymph nodes from two patients with lymphosarcoma. Chromosomal abnormalities in short term cultures of lymphosarcoma and other malignant lymphomas were found by Spiers and Baikie [9] and by Kajii *et al.* [13]. Stewart *et al.* (10) found the same pseudodiploid karyotype in direct preparations and in cells cultured for 4 weeks obtained from Burkitt's lymphoma. Rabson *et al.* [14] established a cell line from the latter tumour and chromosome counts in the cell line varied from 40 to 77 with a modal number of 46 and pseudodiploid karyotype which was similar to that described by Stewart *et al.* [10]. More recent studies have confirmed the presence of chromosome abnormalities in several cell lines established from Burkitt lymphomas [19, 20].

A special role has been claimed for chromosomes 17 and 18 by Spiers and Baikie [21] in a review of cytogenetic studies of reticuloendothelial neoplasms but these chromosomes appeared normal in our cases. Manolov used the increased accuracy of chromosome identification by quinacrine fluorescence banding and found the origin of certain marker chromosomes and other changes but in most Burkitt tumours the chromosomes appeared normal. These special techniques were not known when our cases were analysed but should provide useful information to compare similar marker chromosomes from different tumours, and search for minute structural rearrangements in otherwise normal karyotypes.

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Prophylaxis of Chemically Induced Benign and Malignant Epithelial Tumors by Vitamin A Acid (Retinoic Acid)

WERNER BOLLAG

Department of Experimental Medicine, F. Hoffmann-La Roche Co. Ltd., CH-4002 Basel, Switzerland

Abstract—*The prophylactic effect of retinoic acid on the development of chemically induced papillomas and carcinomas of the skin of mice was examined. The tumors were induced by local application of dimethylbenzanthracene and croton oil. Retinoic acid—200 mg/kg every 14 days—was administered orally during the promotion phase of carcinogenesis. It delayed the appearance, retarded the growth and lead to regression of papillomas. The induction of carcinomas was also inhibited. By prophylactic administration of retinoic acid the appearance of carcinomas was delayed and their incidence reduced.*

INTRODUCTION

RECENTLY it has been shown that systemically applied high doses of retinoic acid have a therapeutic effect on established skin papillomas and carcinomas induced by 7,12-dimethylbenz(α)anthracene and croton oil in mice [1,2,3]. The purpose of the present investigation was to find out whether retinoic acid, when given preventively, has an influence on the induction of skin papillomas and carcinomas.

MATERIAL AND METHODS

Female Swiss albino mice of the random bred Füllinsdorf strain were fed the mouse diet Nafag 199, containing 2500 i.u. of vitamin A per kg of food. Mice were housed in individual cages. The mice weighed 20 to 22 g at the beginning of the experiment.

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Induction of skin papillomas and carcinomas

150γ of 7,12-dimethylbenz(α)anthracene (DMBA) in 0·2 ml acetone were applied twice (on days 1 and 8) on a 5 cm^2 area of shaved skin on the back of mice. From day 38 on, 0·5 mg of croton oil dissolved in 0·2 ml of acetone were applied on the skin twice a week until the end of the experiment or the death of the animals respectively. From day 113 on each animal was carefully examined at intervals usually of 2 weeks until the end of the experiment. The appearance and the course of development of papillomas and carcinomas were recorded. The number of tumors as well as the size of each tumor were determined. The characteristics of papillomas and carcinomas have been described in a former paper [3]. The volumes of the papillomas were calculated with the formula $4/3r^3\pi$, r being the measured mean radius. The mean number of papillomas per animal and the mean volume of papillomas per animal were determined.

Prophylaxis

Two groups of the mice subjected to the treatment with DMBA and croton oil were formed. The control group comprised 44, the group treated prophylactically with retinoic acid consisted of 43 mice. The latter were given doses of 200 mg/kg of retinoic acid as a 1% water miscible solution, containing as solubilizers 8% of Cremophor® and 10% of propylene glycol. A quantity of 0·4 ml of this solution per 20 g mouse were instilled by stomach tube at intervals of 14 days from day 44 on until the end of the experiment. The control group received only the vehicle in the same amounts and on the same days.

RESULTS

Toxicity of retinoic acid

Retinoic acid in the dose of 200 mg/kg administered orally every 2 weeks did not produce weight loss or any severe toxic symptoms. A few mice suffered from partial alopecia and desquamation of the skin. No bone fractures were seen macroscopically.

From the following data it can be deduced that retinoic acid had rather a favorable than a detrimental effect on the survival rate. In the control group 25 of 44 animals survived the experiment, whereas in the retinoic acid-treated group 32 of 43 mice survived. In the control group 8 of 44 animals died with a carcinoma. In the retinoic acid group only 4 of 43 animals died with a carcinoma. As far as the question of toxicity is concerned, it is important to indicate the number of animals which died from causes other than carcinoma. In the control group, these comprised 11 of 44 mice, in the treated group only 7 of 43. This means that retinoic acid in the doses used did not lead to a drug-induced lethality.

Papillomas

The effect of retinoic acid has been determined by counting the number and measuring the volume of the papillomas. For the first time on day 113, i.e. almost 4 months after the first application of the carcinogen, the papillomas were measured. At that time they had reached a size which could easily be determined.

1. *Number of papillomas* (Table 1). As can be seen from Table 1 in the control group the mean number of papillomas per mouse was 4·52. On the other side, in the retinoic acid treated group, only 3·37 papillomas per mouse appeared up to this date. This comparison shows that retinoic acid delayed the appearance of papillomas. In the further course of the experiment in the control group the mean number of papillomas per animal did not change to a great extent: the average of these mean numbers was $\bar{n} = 4{\cdot}76$ with a standard deviation $s = 0{\cdot}46$. In the treated group, however, we observed a significant decrease ($\alpha < 0{\cdot}001$) in the number of papillomas, being 3·37 on day 113 and decreasing to 1·81 at the end of the experiment. At the end of the experiment, 288 days after the first application of the carcinogen, the difference between the 2 groups is still more distinct than at the date of the first measurement of papillomas on day 113. In the control group, the mean

Table 1. *Results of prophylactic treatment with retinoic acid on papilloma induction. Total number and mean numbers of papillomas per animal.*

	Controls			Retinoic acid treated mice		
Day	No. of mice	Total No. of papillomas	Mean No. of papillomas per mouse	No. of mice	Total No. of papillomas	Mean No. of papillomas per mouse
1	44	0	0	43	0	0
113	44	199	4·52	43	145	3·37
127	44	227	5·16	43	154	3·58
148	43	229	5·33	42	143	3·40
162	42	227	5·40	41	130	3·17
176	41	201	4·90	41	121	2·95
197	39	186	4·77	38	103	2·71
211	38	171	4·50	37	96	2·59
225	38	168	4·42	36	66	1·83
239	35	163	4·66	35	69	1·97
253	32	121	3·78	34	70	2·06
274	28	127	4·54	34	73	2·15
288	25	129	5·16	32	58	1·81

Table 2. *Results of prophylactic treatment with retinoic acid on papilloma induction. Total volume of papillomas and mean volume of papillomas per animal.*

	Controls			Retinoic acid treated mice		
Day	No. of mice	Total volume of papillomas (mm^3)	Mean volume of papillomas per mouse (mm^3)	No. of mice	Total volume of papillomas (mm^3)	Mean volume of papillomas per mouse (mm^3)
1	44	0	0	43	0	0
113	44	2766·7	62·9	43	1714·3	39·9
127	44	4644·8	105·6	43	2392·8	55·6
148	43	7249·2	168·6	42	2705·4	64·4
162	42	8757·2	208·5	41	2267·7	55·3
176	41	7081·6	172·7	41	1930·0	47·1
197	39	7652·4	196·2	38	2429·5	63·9
211	38	8145·1	214·3	37	2102·2	56·8
225	38	10211·7	268·7	36	2654·1	73·7
239	35	7677·5	219·4	35	3090·3	88·3
253	32	7752·9	242·3	34	3507·6	103·2
274	28	9216·4	329·2	34	4156·3	122·2
288	25	12985·7	519·4	32	3456·3	108·0

number of papillomas is 5·16, whereas in the retinoic acid treated group the corresponding value is only 1·81. Retinoic acid thus does not only delay the appearance but also reduces the number of papillomas during the course of the experiment. This implies a complete regression of a number of papillomas.

2. *Volumes of papillomas* (Table 2). The total volume of papillomas cannot be used as a parameter for comparison, as the number of animals is decreasing during the experiment in consequence of lethalities caused either by carcinomas or other conditions. The mean volume of papillomas per animal, however, is considered as a reasonable parameter for the evaluation of the results. On the first date registered—113 days after the first application of the carcinogen—the mean volume per animal of the control group was 62·9 mm^3 and thus markedly higher than that of the treated group, this latter being 39·9 mm^3. The control group showed an increase in the mean volume of papillomas per mouse from 62·9 mm^3 on day 113 to 519·4 mm^3 at the end of the experiment. The treated group revealed an increase of the mean volume which was markedly smaller. It rose from 39·9 mm^3 to 108·0 mm^3. Thus, at the end of the experiment, the mean volume of papillomas per mouse of the control group was almost 5 times higher than that of the treated group. For better illustration the values of the mean volume of papillomas per animal ($\bar{V}$) have been analysed and plotted as a function of time (T) on Fig. 1. From these data can be seen that the increase in papilloma volumes is significantly retarded in the retinoic acid treated group compared with the controls. The statistically adapted straight lines (regression lines) are as follows: Controls: $\bar{V} = -108{\cdot}23 + 1{\cdot}65 \times T$. Retinoic acid treated mice: $\bar{V} = -6{\cdot}97 + 0{\cdot}39 \times T$. Result of the t-test: $t = 2{\cdot}93$ with $n = 901$ leads to $\alpha < 0{\cdot}01$.

Carcinomas (Table 3)

There was a difference in the appearance of carcinomas between the control group and the retinoic acid treated group. In Table 3 the cumulative number of carcinomas is indicated. At the end of the experiment, on day 288 after the first treatment with the carcinogen in the control group 18 carcinomas had appeared, whereas in the treated group only 6 were observed. The 18 carcinomas of the control were distributed among 14 mice. Eleven mice

Table 3. *Results of prophylactic treatment with retinoic acid on induction of carcinomas. Incidence of carcinomas in controls and in retinoic acid-treated animals. Figures indicate the cumulative number of carcinomas.*

Days after first treatment with carcinogen	113	127	148	162	176	197	211	225	239	253	274	288
Controls. Number of carcinomas	0	0	2	3	8	9	11	14	15	15	17	18
Retinoic acid treated mice. Number of carcinomas	0	0	0	0	2	4	4	5	5	5	6	6

had only one carcinoma, 2 animals had 2 and 1 mouse had 3 carcinomas. In the treated group 6 mice had a single carcinoma; no mice were observed with more than one carcinoma. As can be seen from Table 3, in the treated group the first carcinomas appeared later than in the control group. On day 148 the first 2 carcinomas were seen in the control group. In the treated group the first 2 carcinomas were diagnosed on day 176. At this time the control groups showed already 8 carcinomas. At the end of the experiment in the control group 8 of the 14 carcinoma bearing animals had died, whereas in the treated group 4 of 6 mice with carcinomas had died. As there was no significant difference in the growth rate of the carcinomas of the 2 groups, these data were not indicated.

Animals free of papillomas and carcinomas

At the end of the experiment only 4 of 25 surviving animals of the control group were free of papillomas and carcinomas. In the retinoic acid treated group, however, 16 of the 32 surviving mice had neither papillomas nor carcinomas.

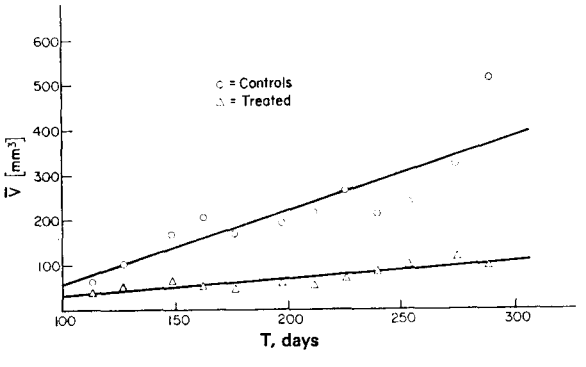

Fig. 1. Mean volume of papillomas per animal in controls [○] and retinoic acid treated mice [△]. ○ = Controls; △ = Retinoic acid treated mice; $\bar{V}$ = Mean volume of papillomas per animal (mm^3); T = Days after first application of carcinogen.

DISCUSSION

The results of the present investigations show that retinoic acid given prophylactically during the promotion phase of carcinogenesis is capable to influence the appearance and development of chemically induced papillomas of the skin. First of all, there is a delay in appearance, which can be considered as a pure prophylactic effect. Secondly, there is a retardation of the growth of the papillomas as can be deduced from the slower increase in papilloma volume per animal in the treated group. Finally retinoic acid induces also the regression of already established papillomas as can be seen from Table 1, showing a reduction of the number of papillomas per animal in the treated group. As already mentioned, the delay of appearance can be explained as a prophylactic effect of retinoic acid. The retardation of the growth and the induction of a regression of papillomas may be considered as caused by a therapeutic effect, as retinoic acid was administered during the whole time of the experiment. This effect has been demonstrated already in previous experiments [1,2,3].

Retinoic acid exerts also a prophylactic effect on the development of carcinomas. The appearance of carcinomas is delayed, as can be seen from Table 3. The incidence is markedly reduced. The number of carcinomas in the treated group is only one third of that of the control group. These results are in good agreement with those of several authors showing that retinol or retinylpalmitate given systemically exert a prophylactic effect in a series of different chemically induced benign or malignant epithelial tumors [4,5,6]. If retinol, retinypalmitate or retinoic acid are given topically, contradictory results have been noticed [7,8,9]. Retinol and retinoic acid differ only little in their chemical structure. Retinol possesses on the same basic structure an alcohol group, retinoic acid a carboxyl group. They have many biological functions and properties in common, e.g. maintenance of growth, protection and differentiation of epithelia and labilization of lysosomes. Thus the prophylactic effect may be based on one of these common properties. It is also possible that retinoic acid is the active metabolite of retinol. This metabolic pathway has been demonstrated [10,11]. Retinol would then exert its prophylactic effect on tumour induction only by its transformation to retinoic acid.

As concerns the mechanism of action of retinoic acid in the prophylaxis of epithelial tumors, only hypotheses can be brought forth. Retinoic acid may be active by its regulatory effect on epithelial tissues, ensuring their growth and differentiation [12]. It may also inhibit the induction of tumors by labilizing lysosomes, a property common to retinol and retinoic acid [13,14,15,16,17]. The increase of lysosomal enzyme activity could help to break down premalignant cells [7].

In previous experimental and clinical investigations, we observed a therapeutic effect of topically or systemically applied retinoic acid on certain premalignant and malignant

epithelial lesions [1,2,3,18,19,20,21]. It is probable that the prophylactic, as well as the therapeutic activity of retinoic acid are based on the same specific property of this compound. Whether the prophylactic effect of retinoic acid on the induction of epithelial tumors in animals has any relevance for the prophylaxis of human premalignancies and malignancies of the epithelial tissues cannot be predicted.

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Announcement

Commencing with Vol. 9, No. 1, the January 1973 issue, this Journal will be published monthly.

Erratum

ABRAHAM KARPAS and ALBERT SAMSO

A new malignant lymphoma induced in the Syrian hamster, *Europ. J. Cancer.* **8,** 231 (1972).

This half-tone was omitted from the above paper.

Figs. 7 *and* 8. *Stained* in vitro *cultured hamster cells obtained from a lymphoma-carrying kidney. Areas with abnormal nuclei and abnormal cytoplasm are shown.*

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